University of Alberta

Association of Vitamin D Status and Vitamin D Receptor Polymorphisms with the Risk and Severity of Acute Lower Respiratory Tract Infection in Infants and Young Children

by

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Abstract

Vitamin D may modulate the host immune response to acute lower respiratory tract infection (ALRI), a frequent cause of hospitalization of children under the age of two years. In a case-control study conducted at the Stollery Children's Hospital in Edmonton, vitamin D status (serum 25-hydroxyvitamin D concentration) was not associated with the risk of early childhood ALRI among children receiving vitamin D in their diets. However, a polymorphism in the vitamin D receptor (VDR) gene was strongly associated with an increased risk of hospitalization for ALRI. These findings suggest that further vitamin D supplementation beyond the amount currently recommended would be unlikely to alter susceptibility to ALRI in the general population. However, the association of a VDR polymorphism and ALRI risk provided evidence of a role for vitamin D in the host response in ALRI that may have relevance to high-risk populations.

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CHAPTER ONE

Background and Rationale

Epidemiology and Pathogenesis of Acute Lower Respiratory Tract Infections in Childhood

The Spectrum of Acute Lower Respiratory Infections in Children

Acute lower respiratory tract infection (ALRI) is one of the most common causes of global mortality and morbidity among children under five years of age, a burden that is largely carried by developing countries[1]. The term ALRI has been adopted by the World Health Organization to encompass the major acute, severe pulmonary illnesses in young children – bronchiolitis (small airways inflammation and obstruction) and pneumonia (purulent alveolar consolidation). Because 'bronchiolitis' and 'pneumonia' are overlapping diagnoses, ALRI refers to the entire range of acute infectious lower respiratory tract diseases to which either term may be applied[2].

Bronchiolitis is recognized in children under the age of two years as a diffuse viral infection of the lower airways, the clinical presentation of which can vary from a mild episode of wheezing with rhinorrhea or nasal congestion to the constellation of more severe clinical findings that may prompt hospitalization – tachypnea, chest retractions, bilateral wheeze and/or crackles on chest auscultation, variable degrees of hypoxemia, and hyperinflation of the lung fields on chest radiograph. Although children under 2 years of age with acute wheezing episodes may eventually develop recurrent wheezing (i.e., asthma), these episodes are almost always triggered by viruses[3] and are thus considered to be ALRIs. Pneumonia is diagnosed when there are symptoms and signs of lower respiratory tract infection (cough, fever, tachypnea, nasal flaring, focal crackles or bronchial breath sounds on auscultation), an absence of diffuse bilateral wheeze, and evidence of pulmonary consolidation on a chest radiograph. Whereas bronchiolitis management is primarily supportive (supplemental oxygen, hydration, and nutrition), pneumonia is often considered to be of bacterial etiology and is routinely treated with oral or intravenous antibiotics. Although croup (laryngotracheobronchitis) is an ALRI, it is excluded from this discussion since it is easily distinguished by hoarseness, cough and stridor and rarely leads to hospitalization due to the availability of effective outpatient therapy[4].

Etiology of Childhood ALRI in North America

Several common viruses and bacteria are causally associated with childhood ALRIs. In the under-five age group, viral pathogens were found to account for up to 90% of all ALRIs in a large US survey[5]. *Streptococcus pneumoniae* remains the most frequent bacterial cause of pneumonia in this age group, yet its incidence is declining due to the widening use of the

pneumococcal conjugate vaccine[6]. The single most common infectious agent associated with pediatric ALRIs in industrialized countries is respiratory syncytial virus (RSV), which is associated with more than half of all ALRI-related hospital admissions in infants and pre-schoolers in the US[5]. Other viruses commonly isolated from nasopharyngeal aspirates in young patients with ALRI include parainfluenza virus, influenza A and B, adenovirus, and the newly-described metapneumovirus[7]. In Canada, the rate of hospitalization for RSV bronchiolitis has increased from 15 to 39/1000 births/year over the past two decades[8]. There is little Canadian data regarding the incidence of childhood ALRI of all etiologies, yet US studies suggest that ALRI accounts for approximately one-fifth of all pediatric hospital admissions in the under-five year old age group[5].

Immunopathogenesis of RSV Bronchiolitis

Consideration of potential interventions to enhance the host response in ALRI requires an understanding of the immunopathogenic cascades induced by the infectious agent. As noted, RSV bronchiolitis is the most common and well-studied ALRI in young children; thus, the following discussion will briefly summarize the current knowledge regarding the pathogenesis of this particular disease.

Respiratory syncytial virus (RSV) is a RNA pneumovirus of the *Paramyxoviridae* family that infects virtually all children at least once, and often twice, by 2 years of age, of whom a small minority require medical care or hospitalization[9]. The virus initially infects the upper respiratory tract epithelium, and may then progress to the lower airway via intercellular spread or aspiration of virus-containing secretions. Despite its name, syncytium formation is rare in humans, yet RSV has some direct cytopathic effects on columnar epithelium, including loss of cilial motility and occasional cell death. However, the peak of illness manifestations does not coincide with the infection itself, but rather with the host cellular response[9].

RSV binds human host cells via a variety of membrane proteins, including toll-like receptor 4 (TLR4) and the chemokine receptor CX3CR1. Viral entry into the cell upregulates STAT and nuclear factor κB (NFκB), both of which trigger the transcription of chemical inflammatory mediators[9]. During the first few days after infection, locally-secreted cytokines including tumor necrosis factor (TNF), interferon-α/-β, and interleukin-8 (IL-8) recruit the cellular effectors of the innate immune response (neutrophils, macrophages, and natural killer cells)[9]. RSV-induced nitric oxide (NO) release by epithelial cells contributes to macrophage infiltration into the airway[10]. RSV may also promote tissue destruction and syncytia formation by upregulating the secretion of tissue-degrading matrix metalloproteinase-9 (MMP-9) by bronchial epithelial cells[11]. Additional cytokine release, antigen presentation, and T cell activation constitute the acquired immune response that develops over the ensuring days. Clinical disease manifestations temporally correlate with the inflammatory response and T cell activation, not with viral replication or antibody production. Notably, specific T cell responses to natural infection do not protect against repeat infections, and in fact, memory T cells do not appear to proliferate upon reinfection[12].

Much of the interest in delineating RSV-related immunopathogenesis has been driven by the epidemiologic association between RSV infection in infancy and childhood asthma, the proposed explanations for which include a differential susceptibility of atopic infants to ALRIs or an effect of RSV infection on the developing immune system[13]. A potential imbalance between the T helper cell type (Th1) and T helper cell type 2 (Th2) arms of the cell-mediated immune response has long been postulated to account for RSV-related immunopathogenesis, in part because it might explain the link to asthma. Animal studies in a variety of species suggested that in severe infections, the acquired T cell response to RSV is guided primarily by Th2-type cytokines (IL-4, IL-5, IL-10, IL-13), leading to eosinophilic pulmonary infiltration, histamine release from mast cells, and IgE secretion[9].

Data from human specimens are less convincing. In vitro data using RSV-stimulated lymphocytes from hospitalized patients with RSV bronchiolitics and non-infected controls revealed a Th1 response predominating in active disease[14]. De Waal et al. similarly found that Th1 (interferon-γ-producing) cells dominated the T cell response to mild RSV infection during the acute phase, but Th2-type cytokines (IL-4 and IL-13) proportionally increased during the convalescent phase[15]. This suggests a 'phenotype switch' that may predispose to the Th2-type responses (e.g., asthma) observed during later childhood following RSV bronchiolitis[16,17].

Some investigators have suggested that the dominance of Th1 cytokines (IFN-γ and IL-12) in mild disease prevents progression to severe disease by inhibiting Th2 proliferation, a theory that is consistent with cross-sectional observations of lower average IFN-γ production in severe RSV bronchiolitis compared to milder disease[18,19]. In one recent study, Pinto et al. showed that Th1 cytokine (IFN-γ and IL-12) secretion by peripheral lymphocytes from infants with mild RSV bronchiolitis was similar to that of cells from a control group of non-infected asymptomatic infants, but there was significantly less secretion of Th1 cytokines by lymphocytes from infants with severe RSV bronchiolitis; Th2 cytokine production did not vary among the three groups[20].

Garofalo et al. measured cytokine concentrations in the nasopharyngeal secretions from infants with three degrees of severity of RSV infection – upper respiratory tract infection (URTI), non-hypoxemic (mild) bronchiolitis, and hypoxemic (severe) bronchiolitis[21]. Similar to Pinto's findings, nasopharyngeal IFN-γ concentrations were significantly higher in mild bronchiolitis compared to severe disease, but Garafalo found that IL-4 concentrations were suppressed in mild bronchiolitis to levels below even those found in asymptomatic controls, strongly corroborating Th1 dominance and Th2 suppression in mild bronchiolitis. There appeared to be a relative resurgence of Th2 activity in severe disease, but the IFN-γ/IL-4 ratio (an estimate of the Th1/Th2 balance) was similar in the severe bronchiolitis and URTI groups, arguing against the hypothesis that a lack of adequate Th2 suppression by IFN-γ is uniquely responsible for severe disease. Further countering the notion of a straightforward relationship between Th2 activity and disease severity, IL-13 and IL-5 (Th2 cytokines) both trended towards lower nasopharyngeal concentrations in severe compared to mild bronchiolitis, and arterial oxygen saturation was not correlated with the concentrations of any of the Th1 or Th2 cytokines[21].

The discrepancies among these studies might be due to the different media in which cytokine concentrations were measured (peripheral lymphocytes versus nasopharyngeal secretions). Furthermore, the causal role of these cytokines remains uncertain. RSV itself may suppress IFN-γ production[22], suggesting that low IFN-γ levels observed in severely-affected patients might be more a marker of viral activity rather than an unsuppressed Th2 response. In fact, animal studies do not implicate IFN-γ as a uniformly protective or pathogenic factor[9].

Pulmonary infiltration by inflammatory cells mediated by a family of secreted mediators called 'chemokines' may be more important than a Th1/Th2 imbalance in RSV-induced immunopathogenesis[23]. CXC chemokines [interferon-inducible protein 10 (IP-10 or CXCL10) and IL-8 (CXCL8)] are chemotactic for polymorphonuclear cells (PMNs), and CC chemokines [macrophage inflammatory protein-1α (MIP-1α or CCL3), monocyte chemotactic protein–1 (MCP-1 or CCL2), RANTES (CCL5)] are chemotactic for monocytes, eosinophils and T cells. McNamara et al. detected high concentrations of chemokines in the lower respiratory tract secretions of infants with RSV bronchiolitis, and found that CXC chemokines (particularly IP-10 and IL-8) were more abundant than CC chemokines (MCP-1 and MIP-1α)[24]. However, in tracheal secretions from infants with RSV bronchiolitis, eosinophil cationic protein (ECP) concentrations were found to be strongly correlated with MIP-1α concentrations, suggesting a role for MIP-1α as an eosinophil chemoattractant or inducer of eosinophil degradation[25].

In the study by Garofalo et al. discussed above, nasopharyngeal MIP-1α concentrations were significantly higher in severe bronchiolitis compared to either mild bronchiolitis or URTI, RANTES concentrations were higher in severe bronchiolitis, and there were significant inverse correlations between oxygen saturation and both MIP-1α and MCP-1[21]. Although Sheeran et al. reported that disease severity in intubated infants were inversely correlated with the concentrations of RANTES and IL-8 in tracheal secretions (but not in nasopharyngeal washes)[26], larger studies have found disease severity to be positively correlated with IL-8 mRNA concentrations in nasopharyngeal samples[27] and plasma IL-8 concentrations[28]. These latter clinical findings are consistent with animal studies, in which depletion or blockade of chemokines generally reduced disease severity[9]. Because CC chemokines stimulate pulmonary eosinophil aggregation and histamine release[23], they may account for at least some of the pathogenic features of severe RSV bronchiolitis that have been traditionally ascribed to Th2 lymphocytes. Somewhat paradoxically, CC chemokine receptors that bind MIP-1α and RANTES are found more frequently on Th1 lymphocytes, which is consistent with the observed dominance of Th1 in acute RSV infection[14].

Successful host management of RSV infection requires effective anti-inflammatory mechanisms to limit autopathogenic lung injury. Counter-regulatory control of chemokine release may be mediated through ligand binding of the peroxisome proliferator activated receptor-γ (PPARγ), a nuclear hormone receptor, which was recently shown to cause dose-dependent inhibition of the synthesis and release of several proinflammatory cytokines and chemokines, including IL-8 and RANTES, from RSV-infected cells[29]. PPARγ ligand activation has broad anti-inflammatory effects through downregulation of proinflammatory cytokine production (including TNF-α , IL-6, MMP-9), as well as possibly through increased

oxidation of proinflammatory arachidonic acid derivatives[30]. In bronchial epithelial cell lines, PPARγ ligand binding reduces MMP-9 secretion via inhibition of a NFκB-mediated pathway[31].

Host factors that govern an individual's innate immune response and Th1/Th2 balance following RSV infection remain unclear. Younger infants (i.e., < 3 months of age) are more likely to experience severe disease, which has been suggested to be due to their tendency towards Th2 responses[32]. Also, preliminary studies suggest that an infant's atopic status may shape the immune response[33,34].

Recently, single-nucleotide polymorphisms (SNPs) in the host genome have been examined for potential associations with RSV infection. SNPs are commonly identified by using restriction enzymes that recognize highly-specific DNA sequences containing polymorphic sites within the gene of interest. These sequences are amplified by the polymerase chain reaction (PCR) and then incubated in the presence of a restriction endonuclease. If a restriction site is present, the enzyme cuts the DNA into two fragments. Visualization of the fragments, the uncut sequence, or a combination of both, permits genotyping.

Increased susceptibility to RSV bronchiolitis has been found in some case-control studies to be associated with SNPs (or haplotypes involving multiple SNPs) in the genes encoding IL-4[35,36], IL-10[37], IL-13[38], CX3C chemokine receptor[39], and CCR5[40] (a chemokine receptor for which the primary ligands are RANTES and MIP-1α). However, other investigators have not detected similar associations with IL-4[38], IL-10[41], or IL-13[36], nor were associations found between susceptibility and SNPs in IL-5[36], IL-8[42], IL-9[37], or TNFα[37]. Increased severity of RSV bronchiolitis has been associated with a specific IL-8 haplotype (comprising six single-nucleotide polymorphisms) that increases transcription of IL-8[43], two IL-10 variants (-1117A/G and -3585T/A[41]), and two TLR4 mutations (Asp299Gly and Thr399Ile)[44], but not with a CD14 SNP[44]. Although these studies point investigators towards those molecules most likely to be implicated in the host response, many problems exist with these gene association studies, including negative findings in potentially underpowered studies, suboptimal control populations (most of the above studies used adult controls), and the possibility of ethnic bias (which most of the studies did not address).

In summary, acute RSV ALRI, at least in its milder form, is characterized by a Th1-dominated immune response, the effectiveness of which may be indicated by the degree to which IFNγ levels are elevated. Although Th2 cytokine predominance during convalescence may be a harbinger of future Th2 tendencies and asthma risk, human data do not wholly support the contention that Th2 cytokines perpetrate severe acute immunopathogenesis. Intriguing observations of pro-inflammatory chemokine activity in respiratory secretions suggest an alternative, or perhaps complementary, mechanism to explain why the host response is responsible for the clinical manifestations of infection. Anti-inflammatory PPARγ-mediated cascades may provide a natural counter-balance to chemokine and tissue-degrading MMP-9 release from the respiratory epithelium. Host genetic polymorphisms likely cause differences in individual susceptibility to RSV infection and disease, but the specific role of SNPs in genes encoding inflammatory mediators such as IL-4, IL-8, IL-10, IL-13, TLR4, CX3C chemokine receptor, and CCR5 have yet to be clarified.

Risk Factors for Childhood ALRIs

Analyses of the epidemiologic risk factors associated with pediatric ALRI in developed countries have focused on RSV-associated disease. Severe RSV infection often occurs in high risk groups (e.g., infants and children born prematurely, or those with congenital heart disease, cystic fibrosis, immunodeficiency states), but the majority of RSV admissions occur in otherwise healthy infants. Risk factors associated with RSV ALRI in otherwise healthy infants include male sex, age less than 6 months, birth during the first half of the RSV season, overcrowding, siblings in the home, and daycare attendance[45]. Lack of exclusive breastfeeding, passive smoke exposure, and lower socioeconomic class may also increase the risk of acquiring RSV infection, although the accumulated data is inconclusive[45]. Family history of asthma in combination with parental smoking and older siblings has been associated with an increased risk of hospitalization for bronchiolitis[46]. In a prospective cross-Canada study of children hospitalized with RSV ALRI, it was found that underlying medical conditions, hypoxia on admission (oxygen saturation < 90%), pulmonary consolidation on chest radiograph, and maternal First Nations or Inuit heritage were independently associated with complicated hospitalization in multivariate analysis[47]. A longer duration of hospitalization for bronchiolitis was found to be associated with a family history of atopy by some investigators[48] but not others[49].

In Canada, the link between socioeconomic disadvantage or geographic isolation and ALRI susceptibility is suggested by rates of pediatric hospitalization for bronchiolitis and pneumonia that are almost four-times higher in the Northwest Territories compared to the Canadian national average[50]. Northern Aboriginal children appear to be at the highest risk of severe bronchiolitis requiring hospitalization of any population in the world that has been studied[51]. The specific reasons for this are unknown – it is not clear whether this represents a clustering of socioeconomic factors (e.g., overcrowding, passive smoke exposure), difficulty accessing health care services early in the disease process, or possibly increased biological susceptibility. Despite the inequitable burden of pediatric ALRI among Canadian Aboriginal children, very few specific public health efforts to explain or reduce the incidence of bronchiolitis and pneumonia in socially-identifiable groups or resource-poor communities have been undertaken. A study of native Alaskan infants with RSV bronchiolitis revealed that breastfeeding was protective against hospitalization for RSV diseases, whereas household overcrowding and underlying medical conditions increased the risk[52]. Risk factors within disadvantaged groups may also be cautiously extrapolated from studies in developing countries, where the risk of severe ALRI in under-fives is increased by younger age, prior history of ALRI, lack of breastfeeding[53], upper respiratory infection in the mother or siblings, severe malnutrition, use of cooking fuel other than liquid petroleum gas, inappropriate immunization for age, and a history of ALRI in a family member[54, 55].

Nutritional Determinants of ALRI Susceptibility and Outcome

Nutritional status has long been linked to the risk of infection-related morbidity and mortality in developing countries, and specifically modified the risk of severe ALRI[56]. Worldwide, malnutrition (i.e., underweight) is considered to be an underlying factor in approximately half of all under-five deaths due to pneumonia[1]. Associations between ALRI

and specific micronutrient deficiencies have been well-described in resource-poor settings where such deficiency states are common[57]. Vitamin A is an evidence-based therapy to reduce pneumonia-specific mortality in children with measles[58], and in developing countries, long-term supplementation with zinc significantly reduces the risk[59] of pneumonia and may also hasten ALRI resolution when used as an adjunctive therapy in hospitalized children[60].

There is uncertainty about extrapolating the effectiveness of these nutritional interventions to target higher-risk populations within developed countries. For example, in one study of Australian indigenous children from remote areas who were hospitalized with ALRIs, neither zinc nor vitamin A supplementation was found to improve the response to treatment and in fact, zinc supplements increased the risk of readmission[61].

Other than breastfeeding, specific nutritional determinants of RSV or bronchiolitis susceptibility or severity have received limited attention. RSV-associated ALRI was found to be *less* common among malnourished than well-nourished children in The Gambia[62], possibly a result of a reduced ability to mount an immune response to the virus, such that viral-induced airway inflammation was less likely to become clinically apparent. In the early 1990s, it was observed that children with RSV ALRI had low serum retinol (vitamin A) concentrations[63]. Given the known benefit of vitamin A in the protection of the respiratory epithelium in the setting of measles infection (a paramyxovirus, similar to RSV), a randomized controlled trial was conducted in the United States to examine whether vitamin A supplementation would reduce morbidity associated with hospitalization for RSV ALRI[64]. There was no therapeutic benefit of vitamin A supplementation; in fact, in children > 1 year of age, supplementation was associated with prolonged hospitalization. In a similar trial in Chile, no overall benefit of vitamin A was observed, yet a subgroup of children with hypoxemia on admission (oxygen saturation < 90%) experienced a significant reduction in the duration of hospitalization[65], suggesting the possibility of a benefit of vitamin A in the most severely-affected patients. The difficulty in accepting the rationale for these studies, particularly in North America, is that there is no epidemiologic evidence of widespread vitamin A deficiency in the population which would explain common susceptibility to RSV infections[66]. Furthermore, serum retinol concentrations are known to be inversely proportional to acute phase reactants (markers of inflammation)[67], suggesting that the low vitamin A levels observed in patients with RSV infection were a result of the infection, rather than a causative factor. The only other micronutrient to be previously considered in RSV susceptibility was in a Turkish study that reported significantly lower serum selenium levels in children with acute bronchiolitis compared to control subjects[68].

Although micronutrient deficiencies are rarely considered amidst the din of public health alarm bells ringing over nutritional excesses and obesity in the developed world, one nutritional deficiency state that continues to be highly prevalent in developed northern regions of the world is vitamin D insufficiency. Given the concurrence of low sunlight exposure (the main source of vitamin D) and higher rates of ALRI at northern latitudes, as well as the growing body of literature proving the immunoregulatory functions of the active metabolite of vitamin D, there is a solid scientific rationale for hypothesizing a role for vitamin D status as a modifiable determinant of the host immune response to ALRI in childhood.

Vitamin D – History and Epidemiology

Adequate supplies of micronutrients are essential for children's normal growth and development[69]. In developing countries, scarce food supplies may lead to micronutrient deficiencies accompanied by protein and calorie malnutrition. Within industrialized countries and contemporary Western society, demographic factors (e.g., poverty, geographic isolation) or unhealthy patterns of food consumption may lead to inadequate intake of some vitamins and minerals, despite an ample supply of macronutrients. In both developed and developing countries, a high prevalence of vitamin D insufficiency (VDI) has become increasingly well-recognized. Although the metabolic bone diseases classically associated with overt vitamin D deficiency (rickets and osteomalacia) have become uncommon in wealthy countries with vitamin D food fortification programs, the long-term adverse consequences of milder VDI have yet to be adequately addressed.

An Historical Perspective on Vitamin D

Rickets, a crippling childhood condition arising from inadequate bone mineralization, is one of the oldest known diseases[70]. However, it was not until the 20th century that a fat-soluble compound in fish liver oil, named vitamin D (because it was the fourth vitamin to be discovered), was found to have dramatic anti-rachitic properties[71]. At about the same time, it was proved that rickets could also be effectively cured by exposing children to sunlight[72], a phenomenon that would later be explained by the discovery that vitamin D3 (cholecalciferol) is endogenously produced in the skin through a reaction catalyzed by the absorption of ultraviolet radiation (UVR).

However, in the early 1900's, children were exposed to declining amounts of sunshine, due in part to worsening environmental air pollution (related to the industrial revolution) as well as lifestyle changes (e.g., spending more time indoors)[73]. Oral supplementation of vitamin D was thus considered to be the most convenient method for treating and preventing rickets, leading to a major public health effort in Canada to increase the vitamin D intake of children. By the 1930s, cow's milk-derived infant formulas sold in developed countries were almost universally fortified with vitamin D[74]. In Canada, the addition of vitamin D to evaporated and dried milk powders was first permitted in 1950[75]. However, in the early 1960s, when any food product could be fortified with vitamin D, concern regarding vitamin D toxicity led to the regulation of vitamin D fortification. When restrictions at that time were felt to lead to a resurgence of rickets, fluid milk was chosen as the most appropriate means of delivering vitamin D to children. In 1975, the fortification of fluid milk with either vitamin D2 or D3 became mandatory across Canada. Currently, Section D.03 of the Canadian Food and Drug Regulations stipulates the limited selection of food products that may be vitamin D fortified, rendering it illegal to incorporate vitamin D into any item not among the following[76]: margarine, infant formulas and adult liquid meal replacement fluids, fluid and powdered milk, and liquid egg products. For infants who are breast-fed (or those not receiving fortified infant formula), the Canadian Paediatric Society, Dietitians of Canada and Health Canada currently recommend vitamin D supplementation[77].

Vitamin D fortification was one of the most dramatic public health successes of the 20th century in North America, reducing the incidence of rickets from one of the most common reasons for hospitalization to a rare occurrence. However, vitamin D-deficiency rickets continues to be a problem in many developing countries, and still occurs infrequently among a minority of immigrant or dark-skinned children in developed countries[78, 79] including Canada[80]. Rickets is possibly becoming more common – in a Canadian Paediatric Surveillance Program (CPSP) study from 2002 – 2004, 104 cases of vitamin D-deficient rickets were reported in the two-year period, including some Caucasian children[81]. Some have argued that the increase in popularity of breast-feeding since the 1970s, with slow uptake of the recommendation to provide vitamin D supplementation to breast-fed infants, has contributed to a recent reemergence of rickets in countries where it was once thought to be virtually eradicated[74].

Vitamin D Insufficiency – A Subclinical Deficiency State

The apparent resurgence of rickets over the past several decades is the tip of an iceberg[82], beneath which exists a much broader problem of potential subclinical vitamin D deficiency in the general population. The detection of a vitamin D-depleted state in the absence of clinical findings has only become possible through the relatively recent establishment of an accurate quantitative biomarker of vitamin D status. 25-hydroxyvitamin D [25(OH)D] is the major circulating form of vitamin D, has a half-life of approximately 3 weeks, and a steady-state serum concentration that reliably reflects the amount of vitamin D derived from both dietary sources and endogenous production over the preceding several months[83]. The 25(OH)D concentration (nmol/L) is measured using high-performance liquid chromatography with mass spectrometry, radioimmunoassays (RIA), and competitive protein-binding assays (CPBA), the latter two being the most common in diagnostic laboratories. Significant discrepancies among the various methods have created challenges in comparing studies and establishing laboratory reference ranges[84]. However, 25(OH)D serum concentrations have not been found to be significantly affected by systemic inflammation[85] in the acute phase response (e.g., malaria[86]) or chronic infection (e.g., HIV[87]), despite potentially-significant alterations in downstream vitamin D metabolism.

Vitamin D status conventionally refers to the 25(OH)D concentration (and is used as such here, unless otherwise specified), such that normal and deficiency states are defined on the basis of biochemical ranges. Severe vitamin D "deficiency" is a serum 25(OH)D concentration at which rickets or osteomalacia develops, which is typically less than 25 nmol/L. "Vitamin D insufficiency" (VDI) is a term commonly used to refer to a suboptimal vitamin D status with adverse long-term health effects, in the absence of rickets or osteomalacia. With the recent coming-of-age in vitamin D research, it was acknowledged that the 'normal range' cannot simply be determined by measuring the average 25(OH)D level in a population in which there is likely a high prevalence of VDI. The threshold that separates insufficiency from 'sufficiency' has therefore only recently been quantified by other means. From an ecological perspective, Vieth has argued that natural experiments of primates or humans with abundant sun exposure prove that 25(OH)D concentrations reaches its steady-state above 120 nmol/L[88]. Of all its adverse effects, poor nutritional vitamin D status is most closely correlated with secondary hyperparathyroidism and reduced

intestinal calcium absorption. Hollis has reviewed the evidence demonstrating that in adults, a 25(OH)D concentration of > 80 nmol/L is necessary to reduce serum parathyroid hormone (PTH) concentration to a physiologic plateau, optimize calcium absorption, and maximize bone mineral density (BMD)[89]. Many clinical laboratories, including the University of Alberta Hospital, now accept that a normal 25(OH)D value for adults is at least 80 nmol/L, but more conservative thresholds (e.g., 40 or 50 nmol/L) are still used in the literature.

A limited number of pediatric studies have addressed the question of defining a normal vitamin D status, but few have involved the large sample sizes necessary to detect inflection points in the inverse relationships between 25(OH)D and PTH, calcium absorption, or BMD in well-defined age groups. Several studies in adolescents have shown an inverse relationship between PTH and 25(OH)D – two of the studies found PTH plateaus, at 25(OH)D > 80 nmol/L in French adolescent boys[90] and > 90 nmol/L in adolescent girls in Cleveland[91], whereas the others did not find obvious plateaus[92,93,94]. Studies in pre-pubertal children have shown similar inverse correlations but without clear inflection points[95,96].

Some investigators have attempted to determine the lower limit of the normal range by using additional biomarkers employed in adult studies such as calcium absorption and BMD. In female adolescents in Finland, linear correlations between BMD and either PTH or 25(OH)D were not apparent, but participants with 25(OH)D < 40 nmol/L had significantly decreased forearm BMD[92]. In a three-year prospective study of peripubertal Finnish girls, the change in BMD was associated with baseline 25(OH)D and none of the participants with baseline 25(OH)D > 50 nmol/L showed losses in lumbar BMD[97]. In midpubertal boys and girls in the southern US, 25(OH)D concentration did not correlate with intestinal calcium absorption, suggesting that the adolescents compensated for decreases in 25(OH)D by increasing PTH secretion and synthesis of 1,25-dihydroxyvitamin D [1,25(OH)2D, the active vitamin D metabolite], evidenced by a significant direct correlation between calcium absorption and both PTH and 1,25(OH)2D[94]. This suggests that with respect to bone mineral metabolism, adolescents may adapt more readily than adults to nutritional VDI, thus obscuring linear associations between vitamin D status, as measured by 25(OH)D concentration, and skeletal outcomes (e.g., BMD).

There is even less known about the normal vitamin D range in young children, infants, or neonates, other than the consensus that vitamin D-deficiency rickets most commonly occurs at 25(OH)D concentrations less than 25 – 30 nmol/L[98]. Vitamin D status in neonates appears to correlate with weight-adjusted whole-body bone mineral content (BMC)[99], but no threshold values can be established because normal BMC ranges are not known. An interesting approach to the determination of normal neonatal levels was taken by Waiters et al. from the University of Alberta, who measured 25(OH)D levels in neonates and their mothers in the Northwest Territories and observed that neonatal values (cord blood samples) were typically 50 – 60% of the maternal level[100]. Therefore, if normal adult levels are > 80 nmol/L, it could be inferred that the normal neonatal concentration is > 40 nmol/L. In one of the only other studies aimed at defining subclinical vitamin D deficiency in neonates, Zeghoud et al. found that newborns with 25(OH)D > 30 nmol/L did not have PTH concentrations above the upper limit of the normal adult range[101], but a plateau was not determined.

In summary, there is evidence suggesting that a subclinical deficiency state exists at all ages, yet there is very little data upon which to establish normative 25(OH)D ranges for children and adolescents. However, for the purposes of discussion, it seems reasonable to consider two definitions of VDI for children and adolescents that are similar to those used in adult studies - a conservative threshold of 40 nmol/L (VDI-C) and an ideal threshold of 80 nmol/L (VDI-I).

Factors that influence vitamin D status

At a population level, the combination of geographic latitude, time of day, and season are unquestionably the most important determinants of vitamin D status[102]. In a landmark study, Webb et al. (1988) demonstrated that human skin did not produce any appreciable quantity of previtamin D3 when exposed to sunlight on cloudless days from November to February in Boston (42.4°N), and from October through March in Edmonton (52°N)[103]. Darker skin colour[104], sunscreen use[105] and clothing of any colour or fabric[106], as well as typical glass and plastic windows, limit the cutaneous production of vitamin D3. Atmospheric pollution absorbs UVB photons and can decrease vitamin D synthesis, as was recently suggested by a study in Delhi, India, which showed that the serum 25(OH)D concentrations of infants and toddlers were inversely related to pollution levels[107].

Additional factors that affect vitamin D status may include gender[108], socioeconomic status[109], obesity[110] and diet[111]. Cholestatic liver disease[112] and renal failure[113] alter vitamin D metabolism, and certain drugs (i.e., phenobarbital, phenytoin, carbamazepine, rifampin) are known to reduce levels of circulating 25(OH)D[114]. In some families, rare genetic mutations in the vitamin D pathways alter serum levels and cellular responses[114].

Prevalence of Vitamin D Insufficiency in Infants, Children and Adolescents

VDI is common throughout the world, most notably in countries located far from the equator[115], and is highly prevalent among healthy Canadian adults[116,117]. In a large sample of US adolescents, VDI was particularly common in girls during the winter, among whom 29% had 25(OH)D levels < 50 nmol/L, and 47% had levels < 62.5 nmol/L[118]. However, sampling was performed at higher latitudes during the summer and lower latitudes during the winter, suggesting that the overall prevalence of vitamin D insufficiency was likely underestimated. A year-round study of adolescents living in Boston found that 24% of participants had VDI [25(OH)D < 37.5 nmol/L], and increased risk of VDI was independently associated with African-American race, winter/spring season, body mass index (BMI), fruit juice consumption, lower milk intake, and lack of exercise, but not gender[93]. There has been only one study of vitamin D status of younger children in the US, which found that only four of 168 girls aged 4 – 8 years living in Georgia were found to have VDI [(25(OH)D < 50 nmol/L][119], a predictable finding given the southern location of the study.

In the only study of vitamin D status in Canadian children and adolescents from the general population, we found a mean 25(OH)D concentration of 47.2 nmol/L (95% CI 43.8 – 50.8 nmol/L) in 68 generally healthy children ages 2 – 16 years who presented to the Stollery Children's Hospital emergency department at the beginning of spring (April 2003). The overall prevalence of VDI [a serum 25(OH)D concentration < 40 nmol/L] was 34%, and 6% of those tested were severely deficient (< 25 nmol/L) despite lacking features of rickets[120].

We found significant associations between vitamin D status and both age and sex. Boys aged 9 to 16 had the highest prevalence of insufficiency (69%), followed by girls aged 9 to 16 (35%), boys aged 2 to 8 (22%), and girls aged 2 to 8 (8%). Overall, participants aged 9 to 16 were at a higher risk for insufficiency than those aged 2 to 8 (OR 5.1, 95% CI 1.7 – 15.6; $P = 0.004$). Among children aged 2 to 8, the mean 25(OH)D concentrations were similar in boys and girls (51.5 and 51.6 nmol/L), whereas among the children aged 9 to 16 years, boys had a significantly lower mean 25(OH)D compared to girls (36.9 versus 48.0 nmol/L).

Prior to the Edmonton study, most of the available Canadian data was related to the unusually high incidence of "nutritional" rickets documented in some northern First Nations communities in the 1980s[121]. A study of eighty mother-child pairs in an Aboriginal community in Manitoba with a high incidence of nutritional rickets revealed a mean 25(OH)D of 26.2 among infants aged 3 to 24 months, of whom 43% had serum 25(OH)D < 25 nmol/L[122]. A study of newborns at delivery in the Northwest Territories found that the mean plasma 25(OH)D concentrations among Indian, Inuit, and Caucasian neonates was 34.1, 34.6, and 41.4 nmol/L respectively; maternal 25(OH)D levels were 52.1, 48.8, and 59.8 nmol/L respectively[100]. More recently, in Winnipeg it was found that 36% of healthy newborns had plasma 25(OH)D < 27.5 nmol/L, but this sample also contained a high proportion of Aboriginal infants[99].

There are several reasons why it is not surprising to find that VDI is common in Canada: 1) the northern latitude of major Canadian cities; 2) aggressive public health efforts that discourage sunlight exposure of children[123]; 3) the decreasing per capita consumption of fluid milk (the major fortified source of vitamin D in the Canadian food supply)[124]; 4) North American children and adolescents are tending to replace milk with beverages such as soft drinks and fruit drinks that do not contain vitamin D[125]; and, 5) current federal legislation limits the categories of food products that can be fortified with vitamin D[126].

Dietary Vitamin D Requirements

In 1995, Health Canada joined the initiative taken by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences (FNB) in the US to develop new 'harmonized' dietary reference intakes (DRI) for Canada and the US. The DRIs for vitamin D, published in 1999, suggest that the 'adequate intake' (AI) for infants, children, and adolescents, male and female, is 200 IU/day (5 mcg/d), and it is specified that this is based on an assumption of a lack of adequate exposure to sunlight[127]. AIs are used to indicate

intakes believed to cover the needs of all individuals in the group where a lack of data prevents authorities from specifying the proportion of individuals who would be appropriately nourished by the suggested amount. Recommended daily allowances (RDAs), which are set to meet the needs of >97% of individuals in a group, are unavailable for vitamin D. For infants who are breast-fed (or those not receiving fortified infant formula), the Canadian Paediatric Society, Dietitians of Canada and Health Canada currently recommend supplements of 400 IU/day of vitamin D supplementation for the entire period of breast-feeding, and 800 IU/day for those infants in northern communities[77].

Although these guidelines are relatively new, the science upon which they were based had changed by the time the guidelines were published. Adult vitamin D requirements have recently been determined through two major lines of investigation – clinical trials evaluating the effect of various vitamin D doses on health outcomes, and pharmacokinetic studies assessing the dose required to raise 25(OH)D serum levels into the normal range (> 80 nmol/L). On both accounts, current guidelines are inadequate[128]. A meta-analysis of vitamin D supplementation for fracture prevention in the elderly found that a dose of at least 700 IU/day is necessary to achieve significant benefit[129]. Based on pharmacokinetic studies, some experts believe that adult doses of at least 2000 IU per day are required in settings of limited exposure to effective sunlight[89], and doses up to 10 000 IU per day are safe[130].

There is no similar data upon which to base infant, child and adolescent dietary recommendations, but the Health Canada/FNB notion that the daily required dose from infancy to young adulthood remains constant is not evidence-based or consistent with other vitamins. In our previous Edmonton study, we found that vitamin D status significantly correlated with weight-adjusted vitamin D intake. Furthermore, all children and adolescents with vitamin D intakes greater than 0.45 mcg/kg/day (18 IU/kg/day) had 25(OH)D serum concentrations above 40 nmol/L[120]. This was consistent with the findings of Vieth et al. who reported that a vitamin D3 intake of at least 25 mcg/day ensured 25(OH)D concentrations greater than 40 nmol/L in healthy Canadian adults[131] (i.e., 0.45 mcg/kg/day for an average adult weighing 70 kg is approximately 30 mcg/day). However, it is unlikely that there is a simple linear association with age or weight, and it is unknown whether this framework can be extrapolated to younger children and infants.

Vitamin D requirements during infancy have been debated for several decades. The debate has largely focused on the vitamin D content of breast milk, which many researchers and pediatricians claim is inadequate to fulfill normal newborn needs[132]. Reports from the 1980s cautioned that exclusively breast-fed infants are at risk of low vitamin D stores[133], particularly if the mother's vitamin D intake is low[134]. Most of the vitamin D activity in human milk is due to 25(OH)D, the concentration of which ranges from 0.74 nmol/L in colostrum to 2.1 nmol/L in mature milk[135]. The approximate overall vitamin D content was thought to be equivalent to 25 IU/L[136], compared to commercial formulas that contain approximately 400 IU/L. However, the vitamin D status of the breast-feeding infant is known to be correlated with the 25(OH)D concentration in the breast-milk[137] and the maternal vitamin D status[138, 139]. Contrary to earlier beliefs that breast-milk is inherently unsuitable as a source of vitamin D for the breast-feeding infant, it was recently shown that the problem is more likely to be maternal vitamin D deficiency. Supplementation of lactating mothers with 4000 IU/day of vitamin D effectively sustained increases in the breast-feeding infant's serum 25(OH)D concentration to > 75 nmol/L[140]. However, postnatal infant supplementation effectively also

leads to increased infant 25(OH)D concentrations[101], and at present is the method most widely employed public health strategy to prevent rickets.

The current Canadian recommendation to provide a daily dose of vitamin D of at least 10 μg (400 IU) to breast-fed infants[77] is an effective measure for the prevention of severe vitamin D deficiency (serum 25(OH)D < 25 nmol/L) and rickets in infants who do not receive adequate sun exposure[141]. However, the benefits of vitamin D supplementation on less overt clinical outcomes are unclear. Among several studies conducted in the 1980 and early 1990s comparing the BMC in vitamin D supplemented and unsupplemented infants at various times during the first year of life, one group showed higher BMC with supplementation[142] while several other groups showed there to be no differences[143,144,145,146]. In studies of term infants, a vitamin D intake of 400 IU/day maintained serum 25(OH)D concentrations greater than 50 nmol/L[147]. Studies involving infants born prematurely have shown that in the context of adequate mineral intake, a daily vitamin D intake of 400 IU, and perhaps as low as 160 IU, may be as effective at stabilizing vitamin D status and normal bone growth as doses up to 2000 IU/day[148, 149, 150]. More recent long-term follow-up studies have reported that vitamin D supplementation during breast-feeding may later lead to higher femoral neck BMC in girls at 8 years of age[151]. The optimal infant dose remains unknown.

Little is known about childhood vitamin D requirements beyond the fact that 400 IU/day of vitamin D reliably prevents vitamin D-deficiency rickets. The extrapolation of adult dietary requirements to children and adolescents is problematic, not only because of age-related differences in vitamin D physiology (e.g., greater amounts of vitamin D may be required during the major growth phases of infancy and adolescence), but also because to date, dietary requirements have been established on the basis of the calciotropic functions of vitamin D in older adults. Whiting and Calvo have argued that functional endpoints unrelated to bone metabolism are needed to effectively establish dietary vitamin D requirements[152].

Vitamin D - Metabolism and Physiologic Actions

Vitamin D has been used therapeutically for over a century, yet its biochemical and physiological attributes have only recently been described in detail (reviewed in Ref. 153), in part because of research efforts driven by the recognition of widespread VDI in adult populations throughout the Western world.

There are two major isoforms of vitamin D consumed or produced by humans: vitamin D2 (ergocalciferol) is found primarily in UV-exposed fungi but has also traditionally been the primary constituent of manufactured vitamin D supplements; vitamin D3 (cholecalciferol) is found in some animal tissues (i.e., liver, sea fish oils, and egg yolks) and is produced endogenously in human epidermis. Although both compounds are found in the natural human food supply, they occur very infrequently and in small quantities. Dietary intake of vitamin D is quantified in term of international units (IU) or microgram (mcg), whereby 40 IU equals 1 mcg. Vitamins D2 and D3 have traditionally been considered to be nutritionally interchangeable, but Vieth and colleagues have clearly demonstrated that vitamin D3 is more

efficient than an equivalent molar quantity of vitamin D2 in its capacity to raise the 25(OH)D serum concentration[154].

Given the scarcity of dietary sources of vitamin D from plant and animal products, the cutaneous reaction stimulated by UVR exposure is undoubtedly the major natural source of vitamin D in humans. The epidermal reaction is initiated when 7-dehydrocholesterol is converted by UVR-induced photolysis to previtamin D3, which then undergoes thermal isomerization to produce vitamin D3[153]. In the mid-1960s, it was determined that vitamin D3 is functionally inert, and must be further metabolized to its active forms. First, vitamin D3 is hydroxylated to form 25-hydroxyvitamin D [25(OH)D], a reaction that primarily occurs in the liver. Since this biochemical step is unregulated, levels of 25(OH)D increase in proportion to vitamin D intake, and thus serum or plasma 25(OH)D levels can be used to assess vitamin D status. A second reaction, by which 25(OH)D is further hydroxylated to form 1,25-dihydroxyvitamin D [1,25(OH)2D], is catalyzed by 1α-hydroxylase. This enzyme is present in the kidneys, although extra-renal production of 1,25(OH)2D may be more significant than previously thought[155], particularly in pregnancy and certain disease states (e.g., granulomatous diseases, rheumatoid arthritis).

In contrast to 25(OH)D production, the synthesis of 1,25(OH)2D is a tightly regulated step that depends primarily on calcium and phosphorus homeostasis. Parathyroid hormone (PTH), whose release from the parathyroid glands is inversely related to the serum ionized calcium concentration, is a major stimulant of 1α-hydroxylase activity. In addition to the PTH-mediated relationship between calcium concentration and 1,25(OH)2D production, there is also an inverse relationship between serum calcium and 1,25(OH)2D that appears to be PTH-independent. Likewise, hypophosphatemia is known to directly stimulate 1α-hydroxylase activity by a PTH-independent mechanism. Insulin and insulin-like growth factor-I (IGF-I) may further enhance 1α-hydroxylase activity, whereas metabolic acidosis dampens the effect of PTH on 1α-hydroxylase, and increases the metabolic degradation of 1,25(OH)2D[153].

Most importantly, 1,25(OH)2D itself is involved in complex feedback regulation of its own synthesis and catabolism, in part by suppressing the synthesis and release of PTH and 1α-hydroxylase. The catabolism of vitamin D metabolites primarily occurs via the oxidation of both 25(OH)D and 1,25(OH)2D in a reaction catalyzed by 24-hydroxylase, an enzyme that is widely distributed in tissues throughout the body and is induced by 1,25(OH)2D in a feedback regulatory cycle. It has also been shown that in humans, high circulating concentrations of 1,25(OH)2D directly stimulate the metabolic destruction of 25(OH)D in the liver[156].

Circulating vitamin D metabolites are almost entirely bound by a serum protein called vitamin D-binding protein (DBP), yet it is the small fraction of free DBP-unbound metabolites which is most accessible to target cells. Because only approximately 5% of total DBP is bound at any given time, DBP is thought to act as a buffer under conditions of vitamin D excess.

It is important to note that despite the rigorous adult data upon which these pathways have been described, there is some evidence that 1α-hydroxylase is not as tightly regulated in

children as it is in adults, leading to elevated 1,25(OH)2D levels following vitamin D supplementation[157]. Although data are scarce, it is likely that the regulation of vitamin D metabolism during the major postnatal growth phases (i.e., infancy and adolescence) differs dramatically from that which occurs in adults or even the more 'quiescent' phases of mid-childhood.

The Vitamin D Receptor

Vitamin D metabolites primarily exert their biologic effects in multiple tissues via the vitamin D receptor (VDR), a steroid receptor present in the cytoplasm of most cells in the body[158]. 1,25(OH)2D is considered to be the active form of vitamin D, since it has 100 times greater affinity for the VDR compared to 25(OH)D. The VDR is widely distributed throughout a variety of tissues, and when bound by 1,25(OH)2D, functions as a transcriptional regulator of a diverse number of genes by its binding to vitamin D response elements (VDREs) in the promoter regions of 1,25(OH)2D-responsive genes[153].

Acting via the VDR, 1,25(OH)2D has anti-proliferative and pro-differentiating properties[159] with widespread physiological effects involving growth and development, endocrine and reproductive functions, and hematopoiesis. Its classic homeostatic VDR-mediated action is the upregulation of dietary calcium absorption from the intestine. Although there are vitamin D-independent mechanisms of calcium uptake, 1,25(OH)2D is the only hormone known to directly stimulate this process.

Just as the roles of various cytokines in RSV immunopathogenesis have been explored through the identification of single nucleotide polymorphisms (SNPs) in the host genome (as described above), the understanding of the role of vitamin D in human health has likewise been improved through the analysis of SNPs in the VDR locus[160]. A functional VDR SNP is revealed by FokI restriction endonuclease activity in the translational start site of the VDR gene[161]. A gene that includes the restriction site (labeled f allele) translates into a VDR that has 427 amino acids, in comparison to the allele that lacks the restriction site (the F allele), which has 424 amino acids. The FokI f allele has been associated with decreased rates of transcription of VDR RNA[162], possibly as a result of a reduction in the efficiency with which the VDR binds to the human basal transcription factor IIB (TFIIB), a general transcription factor that plays a role in mediating the binding of RNA polymerase II to transcription initiation sites on DNA[163].

Another VDR variant is detected by the TaqI restriction endonuclease, with the presence of the restriction site referred to as the t allele and the absence of the restriction site, the T allele[164]. Unlike the FokI polymorphisms, the protein products of TaqI alleles do not differ with respect to amino acid sequence. However, the TaqI TT genotype (relative to the tt genotype) has been shown to be associated with higher levels of VDR expression in peripheral blood mononuclear cells[165].

Allelic distributions vary greatly among ethnic groups. For example, among Caucasian Canadian women, the prevalence of the TaqI genotypes (TT 37%, Tt 47%, and tt 16%)[166] are very similar to northern Europeans (TT 39 %, Tt 46%, tt 16%)[167] and West Africans (TT

45%, Tt 43%, tt 12%)[168], but very different from Peruvians of mixed Spanish and indigenous heritage (TT 83%, Tt 17%, and tt 1%)[169] or Cambodians (TT 91%, Tt 8%, tt 1%)[170]. Similarly, the Peruvians of mixed lineage have a very different distribution of FokI genotypes (FF 7 %, Ff 36 %, ff 57 %)[169] compared to Northern Europeans (FF 42%, Ff 43%, and ff 16%)[167] or Gujarati Indians (FF 64%, Ff 34%, ff 2%). This suggests the possibility that as humans migrated to Europe and North America several thousands of years ago, there may have been natural selective pressures on vitamin D-related genes that allowed some populations to adapt to areas where there was reduced exposure to ultraviolet radiation (e.g., northern parts of North America).

Immunoregulation by 1,25(OH)2D

The non-calciotropic functions of vitamin D have recently received significant attention. Among the best-studied non-calciotropic phenomena are the effects of vitamin D on cellular proliferation and differentiation in the immune system.

The activated hormonal form of vitamin D, 1,25(OH)2D, modulates the activity and cytokine production of cells involved in both the innate and specific responses. As a general rule, 1,25(OH)2D exerts an anti-inflammatory effect by suppressing cellular proliferation and promoting differentiation; for example, it suppresses the proliferation of promyelocytes and promotes their differentiation into monocyte and macrophages[171], and likewise inhibits T cell proliferation while stimulating differentiation into specific T cell subsets[172]. However, there are important exceptions to this rule – 1,25(OH)2D suppresses the differentiation and maturation of monocyte-derived dendritic cells, the most potent type of antigen-presenting cell (APC), leading to the non-specific reduction of T cell responsiveness[173] and impairing the ability of dendritic cells to migrate in response to inflammatory chemokines (CCL4, CCL19)[174]. 1,25(OH2D has been shown in several studies to have other broad anti-inflammatory effects mediated by the suppression of pro-inflammatory cytokines, including TNFα. Furthermore, the expression of several chemokines implicated in the RSV-induced inflammatory response (notably CCR-5 and MIP-2[175]) was significantly suppressed by the administration of vitamin D3 analogues in mice[176].

However, to postulate a role for vitamin D in the immune response against respiratory pathogens, it is important to consider its influence on the relative development of Th1/Th2 cell subpopulations. Dramatic suppression of experimental autoimmune diseases by 1,25(OH)2D (e.g., relapsing encephalomyelitis, a model for multiple sclerosis[177], murine inflammatory bowel disease model[178]) is thought to be mediated primarily by inhibition of Th1 cytokines[179], observations that have driven the theory that activated vitamin D suppresses Th1 and enhances Th2 responses. Indeed, in naïve mouse T cell culture, 1,25(OH)2D preferentially enhanced the development of Th2 cells (cells producing IL-4, IL-5, or IL-10) and inhibited Th1 cells (IFNγ producers), an effect that was primarily mediated by the induction of IL-4 synthesis[180].

Similar results have been observed in experiments using human cell lines. In adult human peripheral blood mononuclear cell (PBMC) culture, 1,25(OH)2D had variable effects on

IFNγ-producing CD4+ T cells (perhaps depending on the individual donor), suppressed IL-2 production and T cell proliferation, increased production of some Th2 cytokines (IL-13, but with only minimal effects on IL-4[181]), and in some experiments, stimulated the production of a unique T-helper cell line that secreted IL-6, a Th2-type cytokine to which both pro-inflammatory and anti-inflammatory properties have been attributed[182,183]. However, in similar experiments performed in cultures of naïve umbilical cord blood T lymphocytes, 1,25(OH)2D led to the balanced inhibition of both Th1 (IFNγ-producing) and Th2 (IL-4 and IL-13-expressing) cell lines, and stimulated the development of the IL-6-secreting subset seen in adult cells (memory T cells), an effect that was augmented by addition of IL-4[184].

The findings in neonatal cells led to further exploration of the possible anti-inflammatory effects of vitamin D on Th2-dominant disease processes in the lungs. In an ovalbumin-sensitized murine asthma model, intraperitoneal injection of 1,25(OH)2D inhibited migration of eosinophils and lymphocytes into the bronchioalveolar lavage (BAL) fluid, reduced IL-4 levels in the BAL, and reduced peribronchiolar inflammation on histology, effects observed even if the 1,25(OH)2D treatment occurred two weeks after induction of the inflammatory response[185]. In a similar experiment, Th2 cytokines were not uniformly increased in the BAL by 1,25(OH)2D, despite elevated IgE and IL-4/IL-13 secretion by circulating ovalbumin-specific T cells[186]. However, others have not reproduced the 1,25(OH)2D-mediated suppression of pulmonary cellular infiltration, and to add a dose of paradox to controversy, VDR knockout mice were unable to develop experimental asthma despite an active systemic Th2 response[187]. These results corroborate the general notion that vitamin D plays both a permissive and inhibitory role in inflammatory regulation, but dispel the assumption that 1,25(OH)2D directly leads to Th2-mediated autopathogenesis. However, further research is required in light of epidemiologic findings of increased risk of asthma, atopic, and allergic rhinitis in Finnish children who received vitamin D during infancy[188].

In addition to the regulation of immune cell proliferation and differentiation, activated vitamin D has also been shown to mediate direct antimicrobial activity by cells of the innate immune response system. Promoters for the genes encoding human cathelicidin antimicrobial peptide (*camp*) and defensin β2 (defβ2) contain VDREs, and administration of 1,25(OH)2D to human keratinocytes, monocytes and neutrophils (but not dendritic cells) induces the expression of these innate antimicrobial peptides[189,190]. Lui et al. recently demonstrated that in activated monocytes, the upregulation of *camp* following toll-like receptor-2/1 (TLR2/1) binding by bacterial peptides is dependent on the activation of the VDR by 1,25(OH)2D, a pathway that requires adequate serum levels of 25(OH)D[191]. However, in what appears to be a negative feedback loop typical of VDR-mediated functions, 1,25(OH)2D was found to downregulate TLR2/TLR4 expression and TNFα synthesis in monocytes via inhibition of NFκB/relA translocation to the nucleus[192].

Many of the initial laboratory observations of antimicrobial activity of 1,25(OH)2D, including the activation of monocytes and the stimulation of cell-mediated immunity, were studied in the context of mycobacterial infection[193]. 1,25-(OH)2D has been shown to imbue human monocytes and macrophages with the capacity to restrict or stop the intracellular growth of *Mycobacteria tuberculosis* [194,195]. Antimycobacterial mechanisms in macrophages may

include the induction of nitric oxide synthase production[196] and the upregulation of camp (cathelicidin) activity[191]. However, as evidence of yet another feedback inhibition route, 1,25(OH)2D has also been shown to limit mycobacterium-induced IFNγ synthesis[197].

The in vitro concentration of 1,25-(OH)vitD3 found to produce anti-mycobacterial effects is considerably higher than normally-circulating concentrations of 1,25(OH)2D. Because 1,25(OH)2D is locally produced by alveolar 1α-hydroxylase function, particularly in the context of granulomatous diseases (e.g., tuberculosis or sarcoidosis)[198], it is thought that 1,25(OH)2D acts primarily via autocrine or paracrine mechanisms, including cross-talk between IFNγ and 1,25(OH)2D signaling. By inhibiting the binding of activated VDR to DNA, IFNγ appears to increase the local synthesis of 1,25(OH)2D by disrupting negative feedback mechanisms[199]. As well, TLR1/2 binding directly causes the upregulation of VDR expression and 1α-hydroxylase activity[191]. These observations imply that markers of systemic vitamin D homeostasis, including the serum 25(OH)D concentration, may not completely reflect vitamin D activity at local sites of inflammation.

Recent clinical studies in humans have further characterized the regulatory role of vitamin D in inflammatory cascades. Recently, it was shown that in adults with congestive heart failure, daily supplementation of 50 μg (900 IU) vitamin D3 for 9 months increased serum concentrations of IL-10 and attenuated an increase in the serum concentration of TNFα[200], observations that are entirely consistent with the dominant anti-inflammatory effects of vitamin D. Timms et al. found that in adult subjects, the concentrations of 25(OH)D and MMP-9 were inversely related, and oral vitamin D supplementation decreased MMP-9 concentrations[201]. As discussed above, tissue matrix metalloproteinases (MMPs) are host enzymes that have been implicated in the promotion of inflammation and tissue damage. As well, the TaqI T allele was associated with diminished production of an anti-proteinase (TIMP-1), a natural inhibitor of MMP-9[201]. These observations suggest that the T allele may predispose to a more severe inflammatory response in diseases such as RSV bronchiolitis, and provide evidence of a potential gene-environment interaction in the regulation of tissue metalloproteinase expression.

In summary, 1,25(OH)2D acts via the VDR to exert potent influences on the immune system. Overall, the effect on the innate system is largely anti-inflammatory through the inhibition of both cellular proliferation and pro-inflammatory cytokine production, but there is also evidence that vitamin D optimizes immune protection against foreign pathogens. With respect to its role in T cell responses, 1,25(OH)2D appears to favour the development of a Th2 response in memory T cells, but its effect on naïve T cells is more balanced; however, there are few clinical observations that extend these laboratory observations.

Vitamin D and Susceptibility to Infection

Consistent with the laboratory findings already discussed, a substantial body of clinical and epidemiologic literature suggests that vitamin D status affects health through immunological pathways that extend beyond those associated with bone metabolism or growth. Vitamin D metabolites are routinely used in the treatment of psoriasis[202], and vitamin D status has been proposed as an etiologic factor in many diseases with seasonal or geographical/latitudinal variations, including multiple sclerosis[203], tuberculosis[204], schizophrenia[205], and prostate cancer[206]. More recently, emphasis has shifted to the role of VDI in the most common chronic diseases in the world, including heart disease, type II diabetes and the metabolic syndrome, and cancer[102]. In fact, one recent analysis proposed that vitamin D supplementation of at least 600 IU/day among North American adults would reduce the incidence of a cancer by about 10 %, with increased benefit if the supplementation dose or UV exposure was higher[207]. A potential advantage of vitamin D supplementation during infancy, beyond the prevention of rickets, is its association with a reduced risk of type 1 diabetes[208,209].

Vitamin D Status and Tuberculosis

The proposed link between vitamin D status and the risk of infectious diseases has been most extensively studied in patients with tuberculosis (TB). Reports from as far back as the middle of the 19th century suggested that cod liver oil was beneficial in the treatment of TB, and therapeutic regimens including calcium, with or without vitamin D, were used until the time that specific anti-TB chemotherapy became available[210].

Epidemiologic studies comparing 25(OH)D serum levels in pre-treatment TB patients and healthy controls have yielded equivocal results. In one recent study of Asians of Gujarti origin, median 25(OH)D levels were significantly lower in untreated TB-infected patients compared to healthy TB contacts, and twice as many TB patients than controls had 25(OH)D levels < 10 nmol/L[211]. An earlier study of patients with Indian subcontinental ethnic origin likewise found lower average 25(OH)D serum concentrations in TB patients compared to healthy matched controls[212], but no difference was found within an Indonesian cohort[213].

Elevated levels of 1,25(OH)2D due to the extra-renal conversion of 25(OH)D to 1,25(OH)2D by activated pulmonary alveolar macrophages might be the cause of hypercalcemia that is observed in 16 % to 48 % of TB cases[214]. Even TB patients without overt hypercalcemia may manifest dysregulated calcium and vitamin D metabolism [215]. The degree of TB-related hypercalcemia is related to vitamin D and calcium intake, which may account for the wide regional variations in the prevalence of hypercalcemia in TB patients [216]. Depressed serum 25(OH)D serum levels coinciding with abnormal elevations of serum 1,25(OH)2D have been observed in some TB patients with hypercalcemia[217], likely as a result of a heightened rate of macrophagocytic and lymphocytic conversion of 25(OH)D to 1,25(OH)2D[218]. It is therefore possible that relatively low serum 25(OH)D serum levels are a result of the disease process instead of a predisposing factor to infection. In addition, the

anti-mycobacterial chemotherapeutic agents isoniazid and rifampicin both individually reduce serum vitamin D levels[219], but this would be unlikely to be a factor in samples collected at the time of diagnosis (as was the case for the above studies). Furthermore, when the drugs are used together and over a long period of time, no significant changes in vitamin D and calcium metabolism are observed with the combination of medications [220,221].

Despite repeated suggestions that vitamin D supplementation may be of benefit in TB therapy, only one study of poor methodological quality has actually been conducted to date[222]. The study involved 24 newly-diagnosed children with tuberculosis, 13 of whom had extra-thoracic disease. Half of the group received standard therapy without vitamin D, the other received standard therapy with vitamin D supplementation (unknown quantity). Using imprecise outcome variables, the latter group was noted to have more obvious clinical improvement and better radiologic findings at 8 weeks of treatment than the group who did not receive vitamin D.

Vitamin D Status and Childhood ALRI

Although rickets has long been associated with the predisposition to bacterial pneumonia[223], specific links between vitamin D status and infectious diseases, other than TB, have not been extensively explored. Over 20 years ago, in a report of a series of Canadian infants sharing the constellation of hepatitis, rickets, hemolytic anemia, failure to thrive and bronchiolitis, termed 'northern infant syndrome', Godel and Hart postulated a potential causative role of micronutrient deficiencies (vitamins A, D, and E)[224], yet the etiology has never been determined even though cases of northern infant syndrome are still occasionally seen.

A recent case-control study conducted in India found that subclinical vitamin D deficiency was a significant independent risk factor for developing severe ALRI in children under five years of age[225]. Specifically, vitamin D insufficiency (VDI), defined as a serum 25-hydroxyvitamin D [25(OH)D] concentration less than 50 nmol/L, was present in 95% of ALRI-affected children, among whom the mean 25(OH)D concentration was 22.8 nmol/L, compared to 61% of control participants who had VDI, with a mean 25(OH)D level of 38.4 nmol/L.

Vitamin D Receptor polymorphisms and Infection

The functional implications of VDR polymorphisms described above may have direct consequences on immunoregulation. Consistent with the notion that the F allele encodes a more efficient VDR, Colin et al. found that in PBMC cultures, the concentration of 1,25(OH)2D required to cause 50% growth inhibition was directly related to the number of f alleles[226]. Similarly, Selvaraj et al. reported that in vitro lymphoproliferation caused by exposure to mycobacterial antigen was more likely to be inhibited by 1,25(OH)2D if the cells expressed the FF or tt genotypes[227]. Overall, these observations suggest that the f allele, and

possibly the T allele, encode a less active VDR and that these SNPs may compromise the host's ability to dampen the inflammatory response.

The relationship between vitamin D metabolism and TB has been recently strengthened by evidence that VDR polymorphisms may influence susceptibility to TB. Individuals with the tt genotype were found to be at a decreased risk of tuberculosis in a study in The Gambia[168]. In contrast, in an Indian cohort, increased TB susceptibility was associated with tt, but only among women[228]. In the aforementioned study of patients of Gujarati origin in the UK, the combination of 25(OH)D < 10 nmol/L and non-tt genotype (TT or Tt) was significantly associated with TB, and individuals bearing the f allele (FokI polymorphism) appeared to have a greater risk of extrapulmonary TB[211].

Among Peruvian adults with pulmonary tuberculosis, we recently showed that both the VDR t and F alleles were associated with faster disease resolution as measured by sputum culture conversion time[169], findings which are consistent with the laboratory studies described above. Genetic variation in the VDR influences susceptibility to a variety of other infectious and non-infectious conditions[229], including reported associations between VDR TaqI polymorphisms and resistance to dengue hemorrhagic fever[230], clearance of hepatitis B virus[168], and leprosy type[231]. There have been no prior studies of associations between VDR polymorphisms and non-mycobacterial ALRI.

In summary, there is substantial laboratory and epidemiologic evidence of gene-environment interactions through which vitamin D mediates the host response to mycobacterial infection. Specifically, low serum 25(OH)D and both the f and T alleles may be risk factors for infectious disease susceptibility and/or progression. Based on the preponderance of laboratory data discussed above, it can be hypothesized that the beneficial actions of 1,25(OH)2D in pulmonary TB are both its anti-proliferative effects on cells involved in the innate response, as well as its role in upregulating phagocytic killing and the release of antimicrobial peptides. Although the effects of vitamin D in other infectious diseases have received little attention, it is plausible that similar phenomena would be observed, particularly in the setting of other respiratory tract infections. Specifically, the clinical manifestations of RSV bronchiolitis are predominantly due to immunopathogenesis, as described above; therefore, a physiologic anti-inflammatory role for vitamin D is plausible. Conversely, given the concern that severe bronchiolitis may be Th2-mediated and that vitamin D supplementation might predispose to Th2-type diseases, the direction of the association remains unpredictable.

Rationale for Study

The findings described above collectively provide strong support for a potential regulatory role of vitamin D in the host response to lower airway infection, yet there are limited clinical data that show that children with vitamin D insufficiency are predisposed to ALRI. A cohort study to investigate the role of vitamin D status in determining ALRI risk would involve a large group of healthy children assessed on a longitudinal basis for both vitamin D status and incidence of ALRI. However, because the overall incidence of severe or hospitalized ALRI is relatively low, this community-base study would require several thousands of children followed closely over a period of several months, entailing very high costs and labour for which funding would have been difficult to obtain without preliminary data. Interventional studies, either using long-term vitamin D prophylaxis or a randomized controlled trial to assess a vitamin D dose administered at the time of hospital admission for ALRI, could also be applied to more conclusively determine the benefit of enhanced vitamin D status on the incidence or severity of ALRIs. However, there is insufficient data to date that establishes the safety or specific dosing regimens of therapeutic doses of vitamin D, or vitamin D analogues, for use in the general pediatric population. Specifically, it is not known whether current daily doses of vitamin D recommended to prevent rickets would be adequate for ALRI risk reduction. If an association between VDI and ALRIs were to be established, further research would be required to determine the appropriate dose and mode of administration of vitamin D for use as either prophylaxis in the prevention of ALRIs, or as adjunctive treatment to reduce the severity of ALRIs.

Therefore, in the present study, we investigated the potential association of hospitalized ALRIs with vitamin D status in children between 1 and 24 months of age using a case-control study design. Recognizing that an individual's *functional* vitamin D status may be affected by the receptor-level response to vitamin D metabolites, we also investigated the potential for associations between ALRI risk and VDR polymorphisms. Among ALRI cases, we further evaluated the association between the severity of the ALRI (duration of supplemental oxygen requirements) and vitamin D status or VDR genotype.

If an association between VDI and ALRI risk, or severity, were to be established, this could lead directly to future interventional studies to determine the role of vitamin D administration in either the primary prevention of ALRIs or in the acute management of ALRIs to reduce disease severity.

Objectives

Primary objectives

To determine whether vitamin D status is associated with the risk of severe acute lower respiratory tract infection requiring hospitalization among infants and young children between 1 and 24 months of age in central/northern Alberta.

Secondary objectives

1. To determine whether vitamin D receptor polymorphisms are associated with the risk of severe acute lower respiratory tract infection requiring hospitalization among infants and young children between 1 and 24 months of age.

2. To explore the potential interactions between vitamin D status and vitamin D receptor polymorphisms (gene-environment interactions) in determining the risk of a severe acute lower respiratory tract infection requiring hospitalization among infants and young children between 1 and 24 months of age.

3. To explore potential associations between vitamin D status or vitamin D receptor polymorphisms and ALRI severity (duration of supplemental oxygen therapy) of acute lower respiratory tract infection among infants and young children between 1 and 24 months of age who are admitted to a tertiary care pediatric centre in Edmonton (cohort study).

4. To determine the prevalence of vitamin D insufficiency and characterize its associated risk factors among infants and young children (aged 1 to 24 months).

5. To describe the demographic and clinical risk factors for hospitalization of infants and young children with acute lower respiratory tract infections.

6. To characterize the demographic and clinical risk factors associated with the severity of disease among infants and young children hospitalized with acute lower respiratory tract infections.

CHAPTER TWO

Vitamin D Status, Vitamin D Receptor Polymorphisms and the Risk of Hospitalization for Acute Lower Respiratory Tract Infection in Young Children: *Objectives and Methods*

Objectives

To determine whether vitamin D status is associated with the risk of severe acute lower respiratory tract infection requiring hospitalization among children aged between 1 and 24 months.

To determine whether vitamin D receptor (VDR) polymorphisms are associated with the risk of acute lower respiratory tract infection (ALRI) requiring hospitalization among infants and young children between 1 and 24 months of age.

Hypothesis

We postulated that children admitted to hospital with severe ALRIs would have a higher prevalence of vitamin D deficiency and a lower mean serum 25(OH)D concentrations compared to healthy control participants. We also predicted that the prevalence of the VDR TaqI T and FokI f alleles would show a tendency to be higher among children admitted with ALRIs compared to healthy controls.

Methods

Study design

This was a conventional case-control study design whereby case and control participants were selected on the basis of the presence or absence of an adverse health outcome (hospitalization for ALRI) and then compared with respect to their exposure to a putative health risk factor (vitamin D status).

Although the case-control design is inherently retrospective, the prospective recruitment at the time of hospital admission for ALRI had several methodological advantages: it increased the likelihood that the case group would be a representative sample of young children and infants admitted with ALRIs; fresh blood samples could be collected for vitamin D status assessment and VDR genotyping; information from parents/caregivers with respect to

potential confounding variables (demographic, clinical and dietary factors) could be systematically collected.

The main advantages of the case-control design were that it was feasible to study a potential ALRI risk factor despite the relative rarity of hospitalization for ALRI within the general pediatric population, and multiple risk factors could be assessed (i.e., vitamin D status and VDR polymorphisms). The main disadvantages of this type of study are that it is only able to produce an estimate of the relative risk, temporal relationships between the variables are uncertain (i.e., it is possible that the host response to an ALRI alters one's vitamin D status), it is only possible to study a single disease outcome (e.g., a cohort study could allow the study of multiple potential infectious disease outcomes of vitamin D insufficiency), there is a possibility of recall bias affecting questionnaire data, and the selection of appropriate controls can be challenging.

Study Setting

Participants were recruited at the Stollery Children's Hospital in the Walter C. Mackenzie Health Sciences Centre, Edmonton, Alberta (latitude 53°N). The Stollery Children's Hospital is the major pediatric centre in Edmonton, with a referral base of 1.6 million people from across northern Alberta, the Northwest Territories, and western Nunavut.

Study Duration

Participants were recruited during two winter seasons: January 1 – March 31, 2005 and January 1, 2006 – March 31, 2006. Human skin does not produce an appreciable quantity of previtamin D3 when exposed to sunlight on cloudless days from October through March in Edmonton[103]. Therefore, in order to consistently assess vitamin D status in all participants prior to the season in which cutaneous vitamin D synthesis occurs, recruitment ended prior to April 1 in each season of recruitment.

Participants

Case Participant Recruitment

Inclusion criteria

Participants recruited to the study were patients between the ages of 1 month (30 days) and 2 years (up to the day prior to second birthday) at the time of admission to the Stollery Children's Hospital general pediatric inpatient ward or pediatric intensive care unit (PICU) with the admitting diagnosis of an acute lower respiratory tract infection (ALRI), including uncomplicated bronchiolitis, pneumonia, or an acute lower respiratory tract infection fulfilling at least one of the case definitions described below ('case definitions'). A 'corrected age' was used for infants born prematurely (< 37 weeks gestation), whereby the

chronological age was considered to start at the date at which the infant would have reached 40 weeks of gestation.

This particular age group was selected because it included virtually all children admitted to the hospital with bronchiolitis, the primary ALRI of interest in this study. Neonates (< 28 days of age) were excluded because the etiology of ALRIs in the neonatal period is usually related to vertical transmission of maternal pathogens (e.g., Group B streptococcus), and thus the risk factors and epidemiology are substantially different from those of older infants and children.

Exclusion criteria

The following criteria were applied to exclude children who were unlikely to have either an uncomplicated viral lower respiratory tract infection (i.e., bronchiolitis) or community-acquired pneumonia:

- A diagnosis of aspiration event upon admission (history of aspiration and x-ray findings consistent with pulmonary aspiration).
- Foreign body aspiration was responsible for the admission
- 'Pulmonary aspiration syndrome': chronic or recurrent aspiration of oral or gastric secretions/contents treated by enteral tube feeding that bypasses swallowing mechanism (nasogastric, nasojejunal, or gastrostomy tube)
- Supplemental oxygen use at home prior to admission for any reason
- Symptomatic congenital heart disease (unrepaired cyanotic heart disease or congestive heart failure)
- Tracheostomy
- Congenital lung abnormality (e.g., hypoplastic or dysplastic lung)
- Chronic interstitial lung disease (e.g., pulmonary hemosiderosis, lymphocytic interstitial pneumonitis)
- Cystic fibrosis
- Neurological disorder that compromises the strength of the respiratory muscles (e.g., spinal muscular atrophy)
- Anatomical abnormality that leads to pulmonary aspiration of oral or gastric secretions (e.g., tracheoesophageal fistula, esophageal web, laryngeal cleft, vocal cord paralysis).
- Congenital or acquired immunodeficiency (e.g., SCID, HIV infection)
- Immunosuppressive chemotherapy within 6 months (i.e., most patients with any recent malignancy)
- Hematologic malignancy (e.g., leukemia)
- Sickle cell disease
- Tuberculosis

Recruitment process

At least three days each week during the study periods, inpatient admission lists were reviewed to identify patients who fulfilled the initial screening criterion of an admitting diagnosis of one of the following:

- Lower respiratory tract infection (including acronyms: e.g., LRTI)
- Upper respiratory tract infection (including acronyms: e.g., URI, URTI)
- Bronchiolitis
- Pneumonia
- Bronchopneumonia
- Pertussis or Whooping cough
- Asthma
- Respiratory distress
- Increased work of breathing (WOB)
- Wheeze
- Cough
- Fever not otherwise specified

Efforts were made to approach all potential participants for possible recruitment within the first 24 to 48 hours of admission. Recruitment was infrequently conducted in the emergency department among patients formally admitted to hospital and awaiting inpatient beds. For each potential participant, inclusion and exclusion criteria were assessed by reviewing the patient's emergency department record and inpatient admission note. Patient eligibility was confirmed by completing the *Case Participant Eligibility Form* (Appendix A).

Case Definitions

In order to differentiate viral ALRIs (bronchiolitis) and bacterial ALRIs (pneumonia), case definitions were developed by consensus among the co-investigators. For all case definitions described below, "tachypnea" was defined by the following respiratory rates (RR):

< 2 months	RR =or> 40
2 - 6 months	RR =or> 35
6 - 12 months	RR =or> 30
12 months - 2 years	RR =or> 25

1. Bronchiolitis:

Although bronchiolitis is a well-recognized clinical syndrome, there is no universal case definition[232]. Consistent with previous descriptions, we defined bronchiolitis as acute-onset lower respiratory tract symptoms of less than two weeks duration in a child less than two years of age, characterized (through history-taking or examination) by all of the following at the time of admission:

1) At least one feature suggestive of a viral respiratory tract infection: rhinorrhea, coryza, cough, or fever.

2) Auscultatory findings suggestive of lower respiratory tract infection: wheeze and/or crackles.
3) At least one feature indicating increased respiratory effort: tachypnea*, intercostal retractions, tracheal tug, supplemental oxygen requirements.

2. Pneumonia:

There is no standard case definition of pneumonia that reliably excludes bronchiolitis, particularly since many physicians use the term 'viral pneumonia' to describe a condition that others might name bronchiolitis. However, for the purposes of classification, we defined pneumonia based on the presence of all of the following criteria that collectively suggest a bacterial ALRI:

1) Temperature > 38.0°C on at least one occasion during the first 24 hours of admission (including in the emergency department)
2) Respiratory distress, as evidence by at least one of the following: tachypnea, accessory muscle use, or nasal flaring noted on admission
3) Focal consolidation or pleural effusion on a chest radiograph performed in a community hospital within 48 hours prior to admission or during admission (except where x-rays were ordered because of suspected aspiration event). Findings are those reported by the radiologist.

3. Respiratory distress not otherwise specified (NOS)

We developed this term to allow for the classification of any patients who did not meet the criteria for either 'bronchiolitis' or 'pneumonia' as defined above, yet had an acute illness (< 2 weeks duration) characterized by the following:

1) Hypoxemia or tachypnea at the time of admission
2) Features of an acute respiratory tract infection, as evidenced by at least one of the following: rhinorrhea, cough, nasal congestion, fever

"Hypoxemia" was defined as an oxygen saturation by pulse oximetry of < 90% on room air at the time of the first assessment recorded in the hospital chart (by emergency medical services or in the emergency department) prior to admission. Given the retrospective nature of data extraction, oxygen saturation by pulse oximetry was considered to be the most objective measure of disease severity available at the time of initial assessment by a health care provider[233].

Control Participant Recruitment

Inclusion Criteria

Control participants were infants and young children admitted to the pediatric day surgery ward for elective surgical procedures, for whom a venous blood sample could be collected at the time of intravenous catheter insertion or during general anaesthesia, and who did not have any history of current or previous lower airway disease (acute or chronic) requiring overnight hospital admission according to parent/caregiver report. The initial protocol

specified selection of controls who were sex- and age-matched (+/- 60 days) to each case participant. However, within two weeks of study initiation, we determined that a matched case-control design was unfeasible based on the slow rate of recruitment of matched controls. Therefore, the protocol was altered to permit recruitment of any potential control participants within the same age range as cases.

Rationale for Selection of Controls

The selection of this particular control population was based on a consensus among the co-investigators that children undergoing elective operative procedures comprised one of the few populations of otherwise healthy children in which the collection of blood samples would be feasible and ethical. Younger age and male sex were considered to be confounding predictors of ALRI susceptibility. Eliminating the matching of cases and controls on the basis of age was especially likely to increase the heterogeneity between the case and control groups. We expected that cases would be younger than controls, since children are less likely to undergo elective surgery in the first few months of life when they are susceptible to ALRI. We considered the lack of sex matching to be a less concerning source of heterogeneity – although male infants may be more susceptible to ALRI, we also expected that more male infants would undergo elective surgery because many elective procedures are more common in boys (e.g., hernia repair, orchidopexy).

Because the study involved venipuncture, the recruitment of healthy children not undergoing intravenous line insertion or general anaesthetic would likely have greatly increased the parental/caregiver refusal rate, thus limiting the feasibility of the study. However, we considered the possibility of a control group comprised of children in the outpatient setting with symptoms of upper respiratory tract infection (URTI) without lower tract involvement. This would have allowed a comparison of the vitamin D status of children with mild and severe respiratory disease. However, in practice, the differentiation between young children who can be treated as outpatients and those admitted to hospital is highly subjective, in part because the diagnosis is imprecise (e.g., many infants considered to have a 'URTI' manifest wheeze, a sign of lower respiratory tract inflammation), and also because of factors unrelated to the child's illness severity at the time of diagnosis, including physician experience and practice patterns, perceived stability of the home situation, and the child's age (i.e., younger infants would be more likely to be admitted because they are perceived to have a greater tendency to clinically deteriorate). These factors would thus have created heterogeneity between the cases and controls that may have been unrelated to the susceptibility of developing ALRI. As well, virtually all children are exposed to the viruses that cause URTI/ALRI during the winter season; therefore, those who develop URTI may share many of the same risk factors as those admitted with ALRI, suggesting that it may have been more difficult to detect differences in risk factors between cases and controls given this design.

Exclusion Criteria

Potential participants were excluded if they had a history of an overnight admission to hospital for 'asthma', 'reactive airway disease', 'bronchiolitis', 'RSV', 'bronchitis', 'pneumonia', 'bronchopneumonia', or 'chest infection' at any time in the past, or fulfilled the

criteria for any of the three case definitions for ALRI (see above) at the time of recruitment. Additional exclusion criteria were identical to those applied to the cases (see above).

Recruitment process

On a weekly basis, a study nurse reviewed operating room slates to identify all patients who possibly fulfilled the initial inclusion criteria. Parents of patients admitted to the pre-operative day ward were approached prior to the surgery. Patient eligibility was confirmed by completing the *Control Participant Eligibility Form* (Appendix B). Inclusion criteria were confirmed by reviewing each patient's hospital outpatient chart to determine age, sex, reason for admission, whether or not the patient will either be having venous sampling or undergo general anaesthesia. Exclusion criteria were determined by review of the patient chart, and discussion with the parent/caregiver prior to the informed consent process to ensure eligibility.

Informed Consent Procedure

Parents or legal caregivers of potential participants were approached at the bedside. The study was explained to the parent/caregiver, including background information, methods, risks/benefits, confidentiality, and freedom to refuse or withdraw. An *Information Sheet* and *Consent form (*Appendix C) was provided to the parent/caregiver. The parent/caregiver was asked if he/she had any questions or needed further time to review the study information. If the parent/caregiver consented to participation, then the *Consent Form* was completed with signatures. Consent to the study but refusal of genetic testing was offered.

Data Collection and Analysis

Primary Outcome Measure

The primary outcome was vitamin D status, as defined by serum 25(OH)D concentration. Comparisons between case and control participant groups involved the consideration of the primary outcome as both a continuous variable expressed as the mean 25(OH)D concentration and a categorical variable (the presence or absence of VDI).

Based on the rationale outlined in Chapter One, the definitions of vitamin D status were established as follows:

- Severe vitamin D deficiency: 25(OH)D < 25 nmol/L
- Vitamin D insufficiency, with a conservative threshold (VDI-C): 25(OH)D < 40 nmol/L
- Vitamin D insufficiency, with an ideal threshold (VDI-I): 25(OH)D < 80 nmol/L

Secondary Outcome Measures and Analyses

The secondary outcome of greatest interest was the distribution of VDR TaqI and FokI genotypes in the case and controls groups. Additional secondary outcomes were demographic and clinical risk factors for ALRI susceptibility assessed by a general questionnaire (participant and family medical history, ethnicity, household factors such as second-hand smoke and crowding, and parental education level), a food frequency questionnaire (FFQ), and weight measurement. In a separate secondary analysis in which vitamin D status was considered as the dependent variable, the same demographic, clinical and dietary factors were considered as potential predictors of vitamin D status.

Sample Size Calculations

Sample size calculations were based on the primary outcome (vitamin D status), expressed as both a continuous and categorical variable.

In our earlier study in Edmonton, the mean serum 25(OH)D concentration in the younger age range (age 2 to 8 years) was 51.5 nmol/L (SD 14.6) and the prevalence of VDI (using a threshold of 40 nmol/L) was 17 %. However, these estimates could not be directly extrapolated to a younger population that would include infants receiving infant formula fortified with vitamin D as well as breastfed infants not receiving any exogenous vitamin D. Because existing Canadian data suggested that the prevalence of vitamin D deficiency was high among young infants[122], we predicted that the mean 25(OH)D concentration would be somewhat lower than 51.5 nmol/L (40 nmol/), and the prevalence of VDI somewhat higher than 17 % (25 %), compared to the 2 to 8 year-old age group.

Given the 25(OH)D interassay coefficient of variation of 12% (for the radioimmunoassay), a difference of 10 nmol/L was considered to be small yet outside the range in which the difference would be likely to be due to lab error. Because of the lack of understanding of the role of vitamin D in early childhood, or the optimal serum concentrations that maximize health benefits as discussed in depth in Chapter One, the purpose of this comparison of means was to test a two-sided alternative hypothesis that even a small difference in vitamin D status existed between cases and controls, perhaps even smaller than that which might eventually be considered to be clinically important. To demonstrate a significant difference between the mean serum 25(OH)D concentration in the control and case groups of 10 nmol/L (i.e., mean 30 nmol/L in the case group), given a standard deviation of 15 nmol/L in both groups (derived from our previous study), 36 participants in each group would provide conventional risks of type I and II errors (80% power and a risk of type I error of 5%).

However, we also planned to have reasonable power with which to detect a significant difference in the prevalence of VDI between case and control groups. For this comparison, the threshold of 40 nmol/L was applied (VDI-C), because it was predicted that the prevalence of VDI-I would be very high in both groups (the prevalence of VDI-I was nearly 100 % in our previous study). We estimated that 25% of healthy controls in the present study would have VDI. In the only study upon which to base estimates, the risk of VDI

among controls was less than half that among cases with ALRIs (61% versus 95 %, using a VDI threshold of 50 nmol/L)[225]. To be relatively conservative and to account for the expectation that the prevalence in our population would be much lower than that encountered in the previous study in India, we planned for a sample size that would allow the detection of a difference between cases and controls if the case group had a prevalence of VDI no greater than double the prevalence in the control group (i.e., prevalence of 25% among controls and 50% among cases). In order to detect this significant difference using Fisher's exact test, with 80% power and a risk of a type I error of 5%, 65 participants were required in each group (130 participants in total). Given only 36 participants in each group, 80% power and a type I error risk 5%, the study would have been powered to detect a difference if the prevalence in the control group was at least 60%.

Recruiting 65 participants to each group was also considered to have the benefit of increasing the power to demonstrate a difference of 10 nmol/L in the mean 25(OH)D concentrations to 97%, thus providing a very low risk of a type II error (the risk of mistakenly rejecting the alternative hypothesis), given a type I error of 5%.

Therefore, in order to accommodate the expression of the primary outcome as both a categorical and continuous variable, we planned to include 65 participants in each group. It was recognized that these calculations were based on imprecise estimates, given the lack of data regarding vitamin D status in the under-two year age group. However, mean 25(OH)D concentrations higher than expected would not affect the power of the study to detect a between-group difference of 10 nmol/L (e.g., a mean of 70 nmol/L among controls and 60 nmol/L among cases would still be considered statistically significant with 97% power and alpha level of 5 %). In contrast, a lower prevalence of VDI could dramatically lower the power (e.g., given 65 participants in each group, a prevalence of VDI of 20 % among cases and 10 % among controls would only have a power of 27% at an alpha level of 5 %).

The study was not powered to detect specific differences in secondary outcomes including VDR genotype distributions, particularly given the complete lack of data upon which to estimate the distribution of VDR alleles in this population. However, we estimated that if the prevalence of the T allele was approximately 85% in the control group (from data discussed in Chapter One), the inclusion of 57 participants in each group provided a power of 80% and risk of type I error of 5 % to detect a difference of 25% between groups (i.e., T prevalence of 60% in the case group).

Feasibility

The Stollery Children's Hospital is a major referral centre for Northern Alberta and parts of northern Canada; therefore, many patients at risk of both ALRIs and VDI are within this referral base. In our experience in a prior vitamin D study involving volunteers in the emergency department, approximately half of all families approached refused to participate. We expected that parents would be more willing to agree to participation to this study because a) unlike our prior study, there was a more direct link between study objectives and the disease for which their child was admitted; b) parents had more time to complete study procedures compared to the busy environment of the emergency department. We thus

estimated that about 30% of parents with eligible children would refuse participation. We estimated that a further 30% of all patients with ALRIs would not be eligible due to exclusion criteria or because blood samples could not be obtained. Therefore, we estimated that approximately 40% of all potential participants would enter the study. In order to recruit 65 case participants, we therefore needed to approach the parents of approximately 163 patients. Because study procedures occurred at the time of recruitment and patient follow-up occurs only during hospital stay, we expected that loss to follow-up or withdrawal would be negligible.

The hospital database showed that there was an average of 182 admissions for bronchiolitis (most responsible diagnosis) in each of 2002-03 and 2003-04 winters (Betty Mah-Pon, Patient Information Services, Capital Health; personal communication). We thus expected the study to be feasible in one season, with a plan to continue to a second year if the sample size was not reached after one season. We did not consider *a priori* early stopping rules, which are rarely addressed in observational research (see Chapter Five).

Data Collection Instruments and Assays

1. Venous Blood Specimen Collection

The venous blood sample was collected from cases by a laboratory phlebotomist. For control participants, the anaesthesiologist or one of the investigators performed the collection at the time of intravenous catheter insertion or by single venipuncture during the course of general anaesthesia. Given the long half-life of 25(OH)D, the specific timing of the specimen collection during the hospital admission was not expected to substantially affect serum concentrations.

Specimens were submitted to the University of Alberta hospital laboratory. Two blood collection tubes were collected for each participant. For measurement of 25(OH)D, a 2 mL sample was collected in a Becton Dickinson Red Top tube. For the VDR assay, a 2 mL whole blood sample was collected in a Becton Dickinson Lavender Top tube (contains EDTA).

2. Vitamin D Status Assessment (primary outcome measure)

Blood specimen collection tubes were centrifuged in the hospital laboratory within 2 hours of collection. The supernatant (serum) was frozen at – 20°C until batched analysis was performed.

Radioimmunoassay (RIA): In the first year of the study, the 25(OH)D serum concentration was determined using the Diasorin/Incstar kit (Stillwater, MN, USA) according to the manufacturer's instructions. This assay is a manual I^{125}-radioimmunoassay (RIA) that has been shown to reliably recover both ergocalciferol and cholecalciferol[234], with an intraassay coefficient of variation of 12% at 60 nmol/L.

Between the two winters during which the study was conducted, the UAH laboratory replaced the RIA with high-performance liquid chromatography and tandem mass spectrometry (LC/MS/MS) as the reference standard method. During an overlap period, lab quality control (QC) assessment demonstrated a consistent, strong correlation between the two methods (Pearson R = 0.87). The linear regression equation estimating the relationship between the two methods was $[25(OH)D]_{LC/MS/MS} = 1.15*([25(OH)D]_{RIA}) - 3.4$, indicating that the RIA slightly under-estimated 25(OH)D concentration, and the degree of negative bias progressively increased with rising concentration (C. Prosser, personal communication).

However, in an attempt to use consistent methodology throughout the study, we initially analysed samples collected in the second year of the study by RIA instead of LC/MS/MS. Because the lab was not performing the RIA assay on a regular basis, all second-year specimens were re-analysed by LC/MS/MS to confirm their accuracy.

LC/MS/MS: This method involved an initial extraction phase and addition of an internal standard to each sample. After thawing serum samples, 50 µL of the internal standard (25-Hydroxyvitamin D3-deuterium-6 at a concentration of 490 nmol/L) and 50µL of a bicarbonate buffer (0.1 mol/L, pH 10.76) were added to 50 µL of each patient serum specimen, control, or calibrator. Samples were incubated in the dark at room temperature for 20 minutes in order to allow the internal standard to equilibrate with vitamin D binding proteins. The proteins were precipitated with 100 µL 2-propanol and extracted with 300 µL hexane. The resulting supernatant was dried and reconstituted in 50 µL methanol. Each solution was transferred to an autosampler vial.

High-performance liquid chromatography (HPLC) was performed using the Agilent Technologies LC system with Supelcosil LC-18 analytical columns. Run time was 6 minutes per sample using a methanol mobile phase. Quantitation was performed using an API 4000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, California, USA) with turbo spray ionization. The following ions were detected: m/z 413.0/395.2 amu for 25-hydroxyvitamin D2, m/z 401.2/383.3 amu for 25-hydroxyvitamin D3, and m/z 407.3/389.4 for 25-hydroxyvitamin D3-D6, the internal standard. The signal intensity for each sample was normalized on the basis of its own internal standard 25(OH)D3-d6 signal, and the concentration was calculated using a standard calibration curve. The total 25(OH)D concentration was the sum of the 25(OH)D2 and 25(OH)D3 concentrations in each sample. Inter-assay coefficients of variation (CV) for 25(OH)D controls were 6% for 25(OH)D3 and 8% for 25(OH)D2.

3. Vitamin D receptor single nucleotide polymorphism (SNP) typing

Analysis of VDR restriction fragment length polymorphisms (RFLP) was performed by the candidate (D.R.) in Dr. Raymond Lai's molecular pathology research laboratory at the University of Alberta Hospital.

Venous whole blood specimens were collected in EDTA tubes and stored at – 20 °C until processing. DNA was extracted from 200 µL whole blood into a final volume of 50 µL of elution buffer using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Mississauga, Ontario).

DNA sequences containing two VDR restriction fragment length polymorphisms were amplified by polymerase chain reaction (PCR) according to methods described previously. The primer sequences for the polymorphic site defined by TaqI restriction endonuclease were 5'-GGAAAGGGGTTAGGTTGGACAGGA-3' and 5'-GGGACGATGAGGGATGGACAGAGC-3', and those for the polymorphic site defined by the FokI restriction endonuclease were 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' and 5'-ATGGAAACACCTTGCTTCTTCTCCCTC -3'.

Amplification was performed in a total polymerase chain reaction (PCR) volume of 25 μL containing 2.5 μL of the extracted DNA solution (0.1 – 0.5 μg DNA), 12.5 μL Hotstar Taq Master Mix (1.25 units HotStar Taq DNA polymerase, 1x PCR buffer with 1.5 mM $MgCl_2$ and 200 μM of each dNTP; Qiagen Inc., Mississauga, Ontario), 2 μL (10 pmol/μL) of each of the appropriate forward and reverse primers (Invitrogen Canada Inc., Burlington, Ontario), and 6 μL of distilled water.

Cycling conditions for all reactions involved an initial 15 minutes at 95 °C to activate the polymerase, followed by 35 cycles at 95 °C for 1 minute, 58 °C for 1 minute, 72 °C for 1 minute (TaqI) or 40 cycles at 95 °C for 1 minute, 58 °C for 1 minute, 72 °C for 30 seconds (FokI). All reactions were completed by a final extension phase at 72 °C for 7 minutes.

The concentration of DNA following PCR amplification was approximately 40 - 45 μg/mL with the FokI primers, and 130 – 170 μg/mL with the TaqI primers, quantified using the Fluoroskan Ascent according to routine methods (Thermo Electron Corporation, Waltham, Massachusetts). The expected amplicons were a 265 bp sequence containing the FokI restriction site and a 717 base pair (bp) sequence containing the TaqI restriction site. Agarose gels (2.5%) were prepared using 75 mL of 1X Tris-Acetate-EDTA buffer, 1.88 mg agarose, and 5 μL (10 mg/mL) ethidium bromide. PCR products were separated by electrophoresis at 100 volts for 45 minutes to confirm the presence of the amplicons prior to digestion. Bands were visualized using the Alphalamager 2200 and AlphaEase FC software (Alpha Innotech Corp., San Leandro, California).

PCR products were then digested in an excess of the FokI and TaqI restriction endonucleases (New England Biolabs Inc, Ipswich, Massachusetts). According to the manufacturer's instructions, 1 unit of either enzyme is the amount required to digest 1 μg of λ DNA in 1 hour at the appropriate reaction temperature. In a total reaction volume of 40 μL, 4 units of the FokI enzyme (1.0 μL of 4 units/μL stock solution) were mixed with 15 μL of PCR products (approximately 0.6 – 0.7 μg DNA) in 4 μL of 10X NEBuffer #4 and 20 μL of distilled H_20. This solution was incubated at 37 °C for 3 hours to ensure the reaction proceeded to completion.

The TaqI reaction contained 4 units (0.2 μL of the 20 U/μL stock solution) of enzyme mixed with 10 μL of PCR products (approximately 1.3 – 1.7 μg DNA) in 4 μL of 10X NEBuffer #3 10X, 0.4 μL (100 μg/mL) BSA, and 25.4 μL distilled H_20 for a total reaction volume of 40 μL. This solution was incubated at 65 °C for 3 hours.

Electrophoresis on 2.5% agarose gels and visualization of DNA bands was performed as described above. Each specimen was assigned one of three distinct banding patterns observed for each polymorphism. According to convention, the presence of a restriction site

is assigned a lower case letter (t, f) and its absence is assigned an uppercase letter (T, F). The TaqI genotypes were:

- TT – 515 bp and 202 bp fragments (due to a conserved TaqI restriction site within the amplicon)
- Tt – 515 bp, 312 bp, and 203/202 bp fragments
- tt – 312 bp, 203/202 bp fragments

The FokI genotypes were:

- FF – 265 bp (undigested amplicon)
- Ff – 265 bp, 195 bp, and 70 bp fragments
- ff – 195 bp and 70 bp fragments

Duplicate genotyping was performed on selected samples to ensure reproducibility. The PCR-RFLP assays and assignment of genotypes was performed without knowledge of the participant identity or group (case or control).

4. *General Questionnaire*

The parent/caregiver of each case and control was guided through the completion of a written questionnaire pertaining to the following demographic, lifestyle and clinical variables (Appendix D):

- Sex
- Date of birth
- Race/ethnicity of biological parents
- Latitude of residence: Edmonton 53° north to Ft. McMurray 56°; north of and including Ft. McMurray to Yellowknife 62°, north of and including Yellowknife
- First Nations living on or off a reserve
- Gestational age at birth
- Birthweight
- Immunization history: up-to-date versus incomplete
- Prior admission to hospital for LRTI
- History of asthma or eczema
- Duration of exclusive breast-feeding
- Birth order among siblings
- Number of household smokers
- Household cooking/heating: electric, gas, wood-burning
- Regular daycare attendance
- Parental or sibling histories of physician-diagnosed asthma
- Parental education levels (primary, high school incomplete, high school completed, university or college incomplete/complete)
- Household crowding: number of adults and children and the number of bedrooms

The completion of the questionnaire was directly facilitated by the study nurse. Several items were explained or clarified in the following manner:

- 'Ancestry' was described as "the people from whom one is descended, often going further back than one's parents";
- 'ezcema' was described as an "allergic-type, itchy, scaly rash" or "very bad dry skin for which a doctor/nurse provided advice or treatment";
- "Up-to-date" immunization status was determined by comparing the patient's age of most recent immunizations to the *expected* age of his/her most recent immunizations based on the Alberta, NWT, or Nunavut immunization schedule. The patient was considered to be 'delayed' if he/she was ≥ 1 month past the appropriate age for missed immunizations;
- 'Exclusive breast-feeding' referred to the number of *completed* weeks of exclusive breast-feeding;
- "birth order" referred to the position of the participant child among the siblings who live in (or grew up in) the same household as the child;
- number of household smokers was counted regardless of how often the person smokes inside the house or around the child;
- an illness contact was someone with a "new cough or cold" that was of less than 2 weeks duration prior to contact with the child;
- family medical history referred only to biological parents and siblings; however, the primary caregivers were considered in the items related to socioeconomic status.

5. Food frequency questionnaire (FFQ)

This FFQ (Appendix E) was adapted from a tool that we recently showed to correlate strongly with both a 24-hour dietary recall conducted by a registered dietitian and vitamin D status[120]. The study nurse and two investigators (DER or ABJ) were trained by a registered dietitian (involved in the prior vitamin D study in Edmonton) to guide the parent/caregiver through completion of the FFQ. For case participants, the FFQ reflected the child's total intake of vitamin D-containing foods in a 'typical' week prior to the onset of the acute respiratory illness. For control participants, the FFQ reflected the child's intake in a 'typical' week preceding the admission.

Weekly vitamin D intake was calculated manually, then divided by seven and expressed as micrograms/day. The vitamin D content of food products was obtained from manufacturers' nutrition labels when available (40 IU = 1 microgram of vitamin D). For natural sources of vitamin D (e.g., ocean fish, eggs, liver), quantities were derived from a published vitamin D database[235].

6. Weight

The participant's weight was measured using a digital scale ('zeroed' prior to each use) and recorded in kilograms on the *Clinical Data Collection Form*. Clothing during weighing consisted

of no more than a diaper and hospital shirt for infants, and underwear, hospital gown and socks for older children. Because participants were recruited throughout various locations in the hospital, it was not feasible to use a single scale. Early in the study it was realized that body length could not be reliably measured in all participants, and it was thus not recorded. Although the lack of length measurements prevented the use of body mass index (BMI) or weight-for-height as anthropometric measures, this decision was not felt to have a major impact on the data analysis. Although measures of macronutritional status may be related to ALRI susceptibility in malnourished populations, they were unlikely to be relevant to the well-nourished children participating in this study. In older children and adults, BMI may be a confounding variable in the assessment of vitamin D status because it is a surrogate marker of adiposity[236], but BMI is not conventionally used to gauge adiposity in young children or infants and thus would not have been relevant in this age group.

Data handling and confidentiality

All questionnaire-based, clinical, and laboratory data were recorded in a MS Excel database. Only investigators directly involved in the study had access to the database. Results of the vitamin D status testing with accompanying management suggestions were delivered to the parents/caregivers of participants through a designated primary care physician. The results of the VDR analysis were not delivered to patients, as it was explained to them during the informed consent process that there is no proven clinical significance of these findings at this time.

Ethical considerations

Informed consent was obtained by parental signature and verbal confirmation of understanding as described above. The study was designed according to the "TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans" and the "Declaration of Helsinki" and was approved by the Human Research Ethics Board (HREB) of the University of Alberta Health Sciences Faculties and the Capital Health Authority.

Statistical analysis

Cases and controls included in the analysis were those with either (or both) a measured 25(OH)D concentration or VDR genotype. The primary outcome measure was the difference between the mean 25(OH)D concentrations of cases and controls. Because of an unusually large negative bias of the year-two RIA values, LC/MS/MS results for year-two samples were preferentially used. In order to combine the datasets from both study years (RIA in year one and LC/MS/MS in year two), the primary outcome analysis included raw second-year LC/MS/MS results and year-one RIA results converted to LC/MS/MS values using the QC linear regression equation as a conversion factor (in order to correct for the slight negative bias of the RIA compared to the LC/MS/MS). Sensitivity analyses were performed using alternative combinations of raw and/or standardized data for year-one and/or year-two samples.

Assuming a normal distribution (assessed by the Kolmogorov-Smirnov test), difference in mean 25(OH)D concentration was assessed for significance by the analysis of variance (ANOVA) for independent samples. The difference between cases and controls with respect to the primary outcome expressed as a dichotomous variable (i.e., the proportion of patients with VDI-C or VDI-I) was assessed by the Chi-square test.

The secondary analysis for potential risk factors for ALRI, and confounding among potential risk factors, included VDR polymorphisms, as well as a variety of additional dietary, demographic, clinical, and anthropometric measures. Bivariate associations were assessed by ANOVA for independent samples, Pearson correlation (R) for continuous variables, and Chi-square or Fisher's exact tests were applied to dichotomous or categorical variables. Results of selected 2 x 2 analyses were reported as crude (unadjusted) or adjusted odds ratios, with 95 % confidence intervals.

Relationships between participant characteristics and vitamin D status were assessed by ANOVA, Pearson correlation, and chi-square tests. Mann-Whitney U test and Spearman rank correlation (ρ) were applied to nonparametric analysis of some non-normally distributed continuous variables. A linear regression model was constructed to determine the independent predictors of vitamin D status.

For the genetic data, deviations from the Hardy-Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$, whereby p is the frequency of the dominant allele and q is the frequency of the recessive allele) and linkage disequilibrium between FokI and TaqI were calculated using the Chi-square statistic. Based on Sasieni's reasoning that allele-based test statistics may not be valid if either the Hardy-Weinberg equilibrium or codominance cannot be assumed[237], analyses of VDR polymorphisms were conducted using genotype as the independent variable. To investigate potential codominance, unadjusted odds ratios were calculated for both homozygotes ($OR_{ff/FF}$) and heterozygotes ($OR_{Ff/FF}$). To explore potential gene-environment interactions, the $OR_{ff/non\text{-}ff}$ was compared between subgroups of participants based on vitamin D status[238].

Logistic regression models were developed to determine the independent predictors of ALRI. The most parsimonious model was established using a backwards step-wise (likelihood-ratio) method including independent variables that had adjusted associations with ALRI ($P < 0.10$). Adjusted odds ratios (OR) were calculated to express the strength of the association between each independent risk factor and the outcome variable (ALRI). Independent variables in the final model were assessed for interactions. Significant interaction terms (unadjusted OR with $P < 0.10$) were added in a forward step-wise fashion to the regression model and retained if statistically significant ($P < 0.05$).

All reported confidence intervals (CI) were at 95% and all reported P values were two-sided; $P < 0.05$ was considered to be statistically significant. In the data tables, P values < 0.05 are highlighted in bold, and P values 0.05 – 0.10 are underlined. Statistical analyses were conducted using SPSS v.10.0 (SPSS Corporation, Chicago, IL).

CHAPTER THREE

Vitamin D Status, Vitamin D Receptor Polymorphisms and the Risk of Hospitalization for Acute Lower Respiratory Tract Infection in Young Children: *Results*

Description of Participants

There were 64 cases and 65 control participants included in the analysis (Table 1). Cases and controls differed with respect to several demographic and environmental characteristics (Table 2).

Table 1. Inclusion and Exclusion of Potential Participants

Cases[1]	Total number of children admitted with ALRI who were eligible for participation and approached for recruitment during the study period	162
	Included	64 (40 %)
	25(OH)D and VDR assays	56
	25(OH)D assay only	8
	Excluded	98 (60 %)
	Parental/caregiver refusal	63
	Specific refusal of blood draw	(14/63)
	Unavailability of a parent/caregiver to provide informed consent	26
	Inability to obtain an adequate blood sample	9
Controls[2]	Included	66
	25(OH)D and VDR assays	61
	25(OH)D assay only	4
	VDR assay only	1
	Excluded (because a blood sample was not obtained despite parental/caregiver informed consent)	13

[1] The age and gender distributions of included and excluded case participants were similar [mean (SD) age: 8.4 versus 7.5 months, $P = 0.358$); gender: 64.1 % vs. 56.7 % boys, $P = 0.413$].

[2] Because the population from whom control participants were drawn was not uniform and subjects were selected by convenience sampling, we did not maintain records of exclusions due to parental refusal. 3/66 control participants were mistakenly recruited despite being < 6 weeks past their second birthday at the time of the blood draw; however, they were included in the analysis because complete laboratory data for these participants had been collected and removal of these participants from the analysis did not change any of the findings or substantially alter risk estimates for primary outcomes (Appendix F-a). None of the control participants had a history of hospitalization for ALRI, nor were any admitted to the SCH for ALRI at any time after recruitment until the end of the study period.

Table 2. Historical and Clinical Characteristics of Cases and Controls

Characteristic	Cases	Controls	*P* value
N	64	66	
Age (months)	8.4 (SD 6.0)	13.2 (SD 6.8)	**< 0.001**
Gender (boys)	41 (64.1 %)	49 (74.2 %)	0.255
Weight (kg)	8.39 (SD 3.0)	9.56 (SD 2.4)	**0.017**
Year of Recruitment Year 1 (2005) Year 2 (2006)	 56 (87.5 %) 8 (12.5 %)	 37 (56.1 %) 29 (43.9 %)	**< 0.001**
Asthma diagnosis in past	4 (6.3 %)	0	0.056
Eczema diagnosis in past	9 (14.1 %)	9 (13.6 %)	0.572
Number of episodes of 'ear infections'	0.53 (SD 1.0)	0.67 (SD 1.9)	0.614
Gestational age at birth (weeks)	38.6 (SD 2.1)	39.1 (SD 2.0)	0.107
Birthweight (grams)	3362 (SD 594)	3348 (SD 620)	0.892
Immunizations are up-to-date for age	49 (76.6 %)	59 (89.4 %)	0.215
Current exclusive breastfeeding	8 (12.5 %)	3 (4.5 %)	0.151
Exclusive breastfeeding index (whereby 1 = 6 months duration, or to present time if < 6 months of age)	0.29 (SD 0.37)	0.42 (SD 0.48)	0.083
Birth order	2.5 (SD 1.3)	2.0 (SD 1.3)	**0.023**
Maternal smoking during pregnancy*	28 (45.2 %)	13 (19.7 %)	**0.002**
Regular daycare attendance (at least one full day per week)*	14 (22.6 %)	12 (18.2 %)	0.345
Exposure to 'sick contact' (someone with cough or runny nose) in the week prior to hospitalization*	49 (79.0 %)	22 (33.3 %)	**< 0.001**
Number of smokers in household*	1.21 (SD 1.4)	0.47 (SD 0.77)	**< 0.001**
Location of Child's Primary Residence South of Edmonton Edmonton and north to Ft. M. Ft..M. and northward	 10 (15.6 %) 54 (84.4 %) 0	 11 (16.7 %) 53 (80.3 %) 2 (3.0 %)	0.363
Maternal ancestry European/Caucasian Aboriginal Other	 37 (57.8 %) 21 (32.8 %) 6 (9.4 %)	 59 (89.4 %) 5 (7.6 %) 2 (3.0 %)	**< 0.001**

Characteristic	Cases	Controls	*P* value
Paternal ancestry			
European/Caucasian	34 (54.0 %)	61 (92.4 %)	**< 0.001**
Aboriginal	23 (36.5 %)	4 (6.1 %)	
Other	6 (9.5 %)	1 (1.5 %)	
Maternal history of asthma*	12 (19.4 %)	11 (16.7 %)	0.585
Paternal history of asthma*	9 (14.5 %)	6 (9.1 %)	0.630
Sibling history of asthma *	14 (22.6 %	6 (9.1 %)	0.074
Maternal education level*			
Post-Secondary Degree Complete	22 (35.5 %)	35 (53.0 %)	0.193
Post-Secondary Degree Incomplete	8 (12.9 %)	7 (10.6 %)	
High School Complete	12 (19.4 %)	13 (19.7 %)	
High School Incomplete	19 (30.6 %)	11 (16.7 %)	
Unknown	1 (1.6 %)	0	
Paternal education level*			
Post-Secondary Degree Complete	18 (29.0 %)	33 (50.0 %)	**0.014**
Post-Secondary Degree Incomplete	7 (11.3 %)	3 (4.5 %)	
High School Complete	14 (22.6 %)	20 (30.3 %)	
High School Incomplete	22 (35.5 %)	10 (15.2 %)	
Unknown	1 (1.6 %)	0	
Crowding index* [(No. adults + No. children) / No. sleeping rooms in house]	1.75 (SD 0.85)	1.25 (SD 0.34)	**< 0.001**
Mode of household heating	(N = 62)	(N = 66)	0.451
Electric radiator	2 (3.2 %)	1 (1.5 %)	
Hot water	6 (9.7 %)	3 (4.5 %)	
Gas furnace (e.g., forced-air)	54 (87.1 %)	61 (92.4 %)	
Wood-burning stove	0	1 (1.5 %)	

Continuous independent variables were summarized by mean and standard deviation (SD), and compared by ANOVA.

* N = 128 due to missing questionnaire data; Ft. M. – Fort McMurray, Alberta

Case-control comparison of 25-hydroxyvitamin D concentrations

In order to assess the acceptability of combining the samples from years one and two, the mean 25(OH)D concentrations by RIA were compared in the control groups. A substantial negative bias was evident in year two relative to year one (e.g., 51.0 nmol/L versus 66.4 nmol/L). Re-analysis of all year-two samples by LC/MS/MS showed strong inter-assay (RIA – LC/MS/MS) correlation (Pearson R = 0.944) but confirmed a strong negative bias of the year-two RIA kit, such that the slope of the linear regression line was much steeper than that of the QC data:

$[25(OH)D]_{LC/MS/MS} = 1.67*([25(OH)D]_{RIA\text{-}YR2}) - 0.006$ (Appendix F-b), compared to

$[25(OH)D]_{LC/MS/MS} = 1.15*([25(OH)D]_{RIA\text{-}QC}) - 3.4$.

LC/MS/MS results were higher than RIA results by a mean difference of +32.1 nmol/L.

Analysis of all year-one samples by LC/MS/MS was not possible, given that lab personnel had accidentally discarded 54 of 93 first-year samples, allowing processing of only 39 first-year samples by LC/MS/MS. Furthermore, inter-assay correlation (RIA – LC/MS/MS) for this group of specimens was only moderate (Pearson R = 0.682), and the linear regression equation showed a weak relationship that differed greatly from those derived from the QC data and the year two dataset: $[25(OH)D]_{LC/MS/MS} = 0.860*([25(OH)D]_{RIA\text{-}YR1}) + 33.266$, suggesting specimen degradation due to prolonged storage (> 1 year) or multiple freeze-thaw cycles. Therefore, LC/MS/MS data from year one could not be used.

The primary outcome analysis was therefore performed using raw year-two LC/MS/MS results and year-one RIA results converted to LC/MS/MS values. Despite the standardization, there remained a difference between the mean 25(OH)D concentrations of the control groups from the two years: year one (N = 37), 72.9 nmol/L (SD 17.2) versus year two (N = 28), 82.9 nmol/L (SD 23.1); $P = 0.049$.

The distribution of 25(OH)D levels approximated a normal distribution (Kolmogorov-Smirnov Z = 0.557; $P = 0.915$); therefore, descriptions and correlations involving the primary outcome measure were analysed using parametric tests. The overall mean 25(OH)D concentration was similar among case and control groups (Table 3). The odds ratio was 1.00 (Table 4), did not measurably change after adjustment for age (Table 4), and there was no significant interaction between age and vitamin D status in the prediction of ALRI risk (OR for interaction term = 1.00, 95 % CI 0.999 – 1.005, $P = 0.293$). Even when the comparison was limited to cases with more severe illness, the mean 25(OH)D among cases was not significantly different from controls (mean among cases with supplemental oxygen requirements for longer than three days: 73.8 nmol/L, $P = 0.407$; mean among cases with hypoxemia at presentation: 73.1 nmol/L, $P = 0.363$). Only 4 of 64 ALRI patients met the case definition for pneumonia, among whom the mean 25(OH)D concentration was 79.4 nmol/L. These patients were included in all further analyses.

The prevalence of VDI-C was very low (Table 3). Most (3/4) of the participants with VDI-C were cases, all of whom were also severely vitamin D deficient (< 25 nmol/L). However, there were no significant differences in the proportions of participants with VDI-C, VDI-I, or severe vitamin D deficiency between the case and control groups (Table 3).

Table 3. Comparison of vitamin D status, vitamin D intake and VDR polymorphisms among case and control participants

Characteristic	Number of participants with available data (Cases, Controls)	All Participants	Cases	Controls	*P* value
25(OH)D concentration (nmol/L)	64, 65	77.1 (SD 22.3)	77.0 (SD 24.2)	77.2 (SD 20.4)	0.960
Prevalence of vitamin D insufficiency (VDI-I) [25(OH)D < 80 nmol/L]	64, 65	70 (54.3 %)	33 (51.6 %)	37 (56.9 %)	0.598
Prevalence of vitamin D insufficiency (VDI-C) [25(OH)D < 40 nmol/L]	64, 65	4 (3.1 %)	3 (4.7 %)	1 (1.5 %)	0.365
Prevalence of severe vitamin D deficiency [25(OH)D < 25 nmol/L)	64, 65	4 (3.1 %)	3 (4.7 %)	1 (1.5 %)	0.365
Estimated dietary vitamin D intake (mcg/d)	64, 62	9.9 (SD 9.6)	11.3 (SD 12.8)	8.4 (SD 4.3)	0.092
Sources of dietary vitamin D intake (mcg/d)					
Infant formula	35, 23	9.8 (SD 12.4)	11.7 (SD 15.5)	6.9 (SD 3.2)	0.145
Fluid milk	22, 42	6.1 (SD 3.5)	7.3 (SD 3.9)	5.5 (SD 3.2)	0.056
Supplement	18,14	7.2 (SD 3.2)	7.1 (SD 3.2)	7.3 (SD 3.4)	0.844
Weight-adjusted dietary vitamin D intake (mcg/kg/day)	62, 61	1.2 (SD 1.1)	1.4 (SD 1.4)	0.95 (SD 0.69)	**0.031**
VDR TaqI genotype[1]	56, 64				
TT		56 (46.7 %)	24 (42.9 %)	32 (50.0 %)	0.736
Tt		56 (46.7 %)	28 (50.0 %)	28 (43.8 %)	
tt		8 (6.7 %)	4 (7.1%)	4 (6.3 %)	

Characteristic	Number of participants with available data (Cases, Controls)	All Participants	Cases	Controls	*P* value
VDR FokI genotype[1]	56, 64				
FF		38 (31.7 %)	14 (25.0 %)	24 (37.5 %)	**0.009**
Ff		66 (55.0 %)	29 (51.8 %)	37 (57.8 %)	
ff		16 (13.3 %)	13 (23.2 %)	3 (4.7 %)	

Continuous independent variables were summarized by mean and standard deviation (SD), and compared by ANOVA.

[1] The allelic distributions of TaqI and FokI polymorphisms did not significantly deviate from the Hardy-Weinberg equilibrium (TaqI: $\chi 2 = 1.69$, $P = 0.429$; FokI: $\chi 2 = 2.28$, $P = 0.319$). TaqI and FokI were in linkage disequilibrium ($\chi 2 = 9.7$, $P = 0.04$).

Because of the lack of a single uniform assay for the measurement of 25(OH)D in all samples from both study years, a sensitivity analysis of the primary outcome was undertaken by applying various approaches to the analysis of raw and/or standardized data (Table 4). Although some approaches suggested a small but significantly different vitamin D status among case and control participants, differences were minimized and rendered statistically non-significant after adjusting for age (Table 4).

Table 4. Sensitivity analyses of primary outcome measure (difference in 25-Hydroxyvitamin D levels between case and control groups)

25(OH)D Measure	N Case, Control	Mean 25(OH)D, nmol/L (SD)		Odds Ratio (95 % CI)	
		Cases	Controls	Crude	Age-adjusted
LC/MS (converted from RIA for year one; raw LC/MS for year two)[1]	64, 65	77.0 (SD 24.2)	77.2 (SD 20.4)	1.00 (0.98 – 1.02	1.00 (0.98 – 1.01)
Standardized RIA (raw RIA for year one; standardized RIA for year two)[2]	64, 65	69.2 (SD 20.9)	70.9 (17.1)	1.00 (0.98 – 1.01)	0.99 (0.97 – 1.01)
Non-standardized RIA – raw RIA from both years one and two	64, 65	66.7 (22.3)	59.7 (15.8)	**1.02 (1.00 – 1.04)**	1.02 (0.998 – 1.04)
Non-standardized RIA – year one only	56, 37	70.6 (20.1)	66.4 (14.9)	1.01 (0.99 – 1.04)	1.01 (0.99 – 1.04)
Non-standardized RIA - year two only	8, 28	38.8 (17.1)	51.0 (12.5)	**0.94 (0.88 – 0.998)**	0.95 (0.89 – 1.01)
LC/MS – second year only	8, 28	71.3 (31.9)	82.9 (23.1)	0.98 (0.95 – 1.01)	0.99 (0.96 – 1.02)

[1] LC/MS results for year one estimated on the basis of RIA results, according to the laboratory quality control (QC) linear regression equation: $25(OH)D]_{LC/MC/MC} = 1.15*([25(OH)D]_{RIA}) - 3.4$.

[2] Year-two RIA data were standardized to year-one RIA by combining the QC and year-two linear regression equations to yield the following correction factor: $[25(OH)D]_{RIA\text{-}QC} = \{1.67*([25(OH)D]_{RIA\text{-}YR2}) + 3.394\}/1.15$. Rendered values were combined with raw year-one RIA data.

Case-control comparison of VDR genotype distributions

The distribution of TaqI polymorphisms was similar among case and control groups (Table 3). However, there was a significantly higher proportion of cases with the FokI ff genotype (and lower prevalence of the FF genotype) compared to control participants (Table 3). The crude odds of ALRI for a child with the ff genotype compared to a child with the FF genotype ($OR_{ff/FF}$) was 7.43 (95% CI, 1.80 – 30.67) and the crude odds of ALRI in heterozygotes ($OR_{Ff/FF}$) was 1.34 (95 % CI, 0.59 – 3.05). Because $OR_{Ff/FF}$ was close to 1 and non-significant, further analyses were performed by comparing ff to 'non-ff' (annotated OR_{ff}).

When participants were sub-grouped into those with and without VDI-I, the OR_{ff} was substantially higher and remained significant among those with VDI-I compared to those with optimal vitamin D status, providing evidence of a possible gene-environment interaction (Table 5). There were too few participants with 25(OH)D less than 40 nmol/L to assess gene-environment interactions involving VDI-C.

Table 5. Gene-Environment Interactions in the Susceptibility to ALRI

	All Participants N= 120		25(OH)D < 80 nmol/L N= 66*		25(OH)D ≥ 80 nmol/L N = 53*	
	ff	Non-ff	ff	Non-ff	ff	Non-ff
Case	13	43	7	22	6	21
Control	3	61	1	36	2	24
OR_{ff}	6.15 (1.65 – 22.89)		11.46 (1.32 – 99.46)		3.43 (0.624 – 18.85)	

* 119/120 participants included in subgroup analysis; 1 participant for whom genotyping was performed did not have 25(OH)D measured and thus could not be included.

There were several differences among the three FokI genotypes with respect to several ALRI risk factors, the following of which were associated with increased odds of the FokI ff genotype versus a non-ff genotype (Table 6): paternal Aboriginal ethnicity, shorter duration of exclusive breastfeeding, maternal smoking during pregnancy, and an increased number of smokers in the household. FokI ff remained significantly predictive of ALRI after adjusting for both maternal and paternal Aboriginal ethnicity by logistic regression (OR_{ff} 5.09; 95% CI, 1.26 – 20.61), adjusting only for maternal smoking during pregnancy (OR_{ff} 4.16; 95% CI, 1.05 – 16.47) and after controlling simultaneously for multiple host and household factors (Table 7).

When only those participants for whom both parents were of European heritage were included in the analysis (N = 80), the association of FokI ff with ALRI remained strong but with wide confidence intervals (OR_{ff} 18.32; 95 % CI, 2.07 – 162.50). There were too few control participants of Aboriginal descent (4 participants) to conduct a separate analysis limited to Aboriginal participants. TaqI polymorphisms were not associated with ethnicity or vitamin D status (Appendix F-c).

Table 6. Associations between VDR FokI polymorphisms and participant characteristics

Characteristic	VDR FokI Genotype				OR (95 % CI)*
	FF	Ff	ff	*P* value	
Mean age (months)	13.2 (SD 7.2)	9.7 (SD 6.7)	10.1 (SD 5.0)	**0.033**	0.98 (0.90 – 1.06)
Gender Boys* Girls	 29 (35.8 %) 9 (23.1 %)	 42 (51.9 %) 24 (61.5 %)	 10 (12.3 %) 6 (15.4 %)	 0.372	 0.78 (0.26 – 2.31)
Maternal Ethnicity Aboriginal* European	 5 (20 %) 28 (31.8 %)	 14 (56.0 %) 50 (56.8 %)	 6 (24.0 %) 10 (11.4 %)	 0.209	 2.68 (0.869 – 8.29)
Paternal Ethnicity Aboriginal* European	 5 (18.5 %) 29 (33.7 %)	 15 (55.6 %) 49 (57.0 %)	 7 (25.9 %) 8 (9.3 %)	 0.053	 **3.23 (1.07 – 9.71)**
Mean 25(OH)D (nmol/L)	72.1 (SD 19.5)	79.4 (SD 24.8)	79.7 (SD 17.6)	0.250	1.01 (0.98 – 1.03)
Mean weight (kg)	9.5 (SD 2.5)	8.5 (SD 2.7)	10.1 (SD 3.3)	0.053	1.18 (0.97 – 1.43)
Median duration of exclusive breast-feeding (index, whereby 1 = 6 months)	0.25 (IQR 0.58)	0.21 (IQR 0.71)	0.00 (IQR 0.2)	0.061	**0.07 (0.01 – 0.78)**
Median birth order among siblings	2.0 (IQR 2.0)	2.0 (IQR 2.0)	2.5 (IQR 0.9)	0.103	1.23 (0.85 – 1.76)
Maternal smoking during pregnancy	12 (32.4 %)	16 (24.6 %)	12 (75 %)	**0.001**	**7.93 (2.36 – 26..65)**

Characteristic	VDR FokI Genotype				OR (95 % CI)*
	FF	Ff	ff	*P* value	
'Sick contact' in previous week	20 (54.1 %)	31 (47.7 %)	12 (75.0 %)	0.327	2.76 (0.83 – 9.17)
Median number of smokers in household	0.0 (IQR 1.5)	0.0 (IQR 1.0)	1.0 (1.0)	**0.015**	**1.52 (1.04 – 2.22)**
Sibling history of asthma	6 (16.2 %)	10 (15.4 %)	4 (25.0 %)	0.548	1.77 (0.51 – 6.19)
Maternal non-completion of high school	9 (24.3 %)	15 (23.4 %)	5 (31.3 %)	0.808	1.46 (0.46 – 4.62)
Paternal non-completion of high school	11 (30.6 %)	16 (24.6 %)	4 (25.0 %)	0.802	0.91 (0.27 – 3.08)
Median household crowding index	1.3 (IQR 0.7)	1.3 (IQR 0.7)	1.3 (IQR 1.1)	0.861	1.04 (0.49 – 2.20)

* Unadjusted odds ratio, whereby an odds > 1 reflects an increased probability of the ff genotype versus a non-ff genotype in the presence of the given ALRI risk factor (for categorical variables where more than one category is listed, the * indicates the risk factor) or a higher value of a continuous variable.

Case-control comparison of dietary vitamin D intake

Total vitamin D intake and weight-adjusted vitamin D intake were significantly higher in cases compared to control participants (Table 3). However, the difference disappeared after controlling for age (Table 7).

Case-control comparison of participant characteristics

Several additional differences between the case and control group participants were apparent (Table 2). Factors that were significantly associated with ALRI included younger age, lower weight, higher birth order among siblings, maternal smoking during pregnancy, exposure to a 'sick contact' during the previous week, the number of smokers in the household, maternal and paternal Aboriginal ancestry, low paternal education level, and a higher household crowding index (Table 2). Additional risk factors of borderline significance ($0.05 < P < 0.10$) included a past diagnosis of asthma, shorter duration of exclusive breastfeeding, and a biological sibling history of asthma (Table 2).

Multivariate analysis of ALRI risk factors

To establish the risk factors that were independently associated with ALRI, binary logistic regression models were constructed. First, because of the significant age difference between the case and control groups, age-adjusted odds ratios were calculated. Other than weight and vitamin D intake per kilogram, which did not remain significant, age adjustment brought all of the other risk estimates closer to 1 (Table 7).

Individual risk factors for ALRI were assessed as potential independent predictor variables after controlling for potentially confounding host and household variables (independence-testing models, Table 7). The parsimonious model (the model with the best predictive capacity and limited to significant independent risk factors) demonstrated that younger age, FokI ff genotype, higher household crowding index, recent sick contacts, and paternal Aboriginal ethnicity were collectively most predictive of ALRI (Table 7). The only significant interaction term between the variables in the final model was 'age x crowding' (OR 0.86, 95 % CI 0.74 – 1.01, $P = 0.070$), yet this term did not remain statistically significant when added to the model (adjusted OR 0.87, 95 % CI 0.73 – 1.04, $P = 0.123$), suggesting that the interaction was accounted for by the other covariates, and was thus not retained.

Many of the ALRI risk factors were not included in the final regression model because of significant confounding. In particular, maternal and paternal Aboriginal heritage were significantly associated with one another, and one or both were strongly associated with several ALRI risk factors – maternal/paternal high school non-completion, maternal smoking during pregnancy, a higher number of smokers in the household, higher household crowding index, higher birth order, shorter duration of exclusive breastfeeding, and lower participant age (Appendix F-d).

Table 7. Association of participant characteristics and ALRI susceptibility (Binary Logistic Regression)

Risk Factor	Crude OR (95 % CI)	Age-adjusted OR (95 % CI)	Independence-testing models[1] OR (95 % CI)	Parsimonious Model[2] OR (95 % CI)
25(OH)D concentration	1.00 (0.98 – 1.02)	1.00 (0.98 – 1.01)	-	-
FokI ff genotype (vs. non-ff)	**6.15 (1.65 – 22.89)**	**6.40 (1.64 – 24.94)**	**6.89 (1.33 – 35.77)**	**10.04 (1.75 – 57.73)**
Vitamin D intake (mcg/kg/day)	**1.78 (1.05 – 3.03)**	1.25 (0.78 – 2.00)	-	-
Age	**0.84 (0.75 – 0.94)**	-	**0.84 (0.74 – 0.94)**	0.93 (0.86 – 1.01)
Weight	**0.85 (0.74 – 0.98)**	1.15 (0.91 – 1.46)	-	-
Duration of exclusive breast-feeding	0.48 (0.21 – 1.11)	0.53 (0.22 – 1.32)	-	-

Risk Factor	Crude OR (95 % CI)	Age-adjusted OR (95 % CI)	Independence-testing models[1] OR (95 % CI)	Parsimonious Model[2] OR (95 % CI)
Birth order among siblings	**1.38 (1.04 – 1.84)**	**1.35 (1.00 – 1.83)**	1.27 (0.87 – 1.85)	-
Maternal smoking during pregnancy	**3.36 (1.53 – 7.37)**	**3.36 (1.46 – 7.71)**	0.77 (0.19 – 3.18)	-
'Sick contact' in previous week	**7.61 (3.36 – 17.22)**	**6.33 (2.72 – 14.74)**	**7.71 (2.64 – 22.52)**	**8.07 (2.69 – 24.24)**
Number of smokers in household	**2.02 (1.34 – 3.04)**	**1.97 (1.28 – 3.04)**	1.92 (0.95 – 3.87)	-
Maternal Aboriginal heritage (vs. non-Aboriginal)	**5.96 (2.08 – 17.03)**	**5.21 (1.77 – 15.40)**	2.99 (0.77 – 11.57)	-
Paternal Aboriginal heritage (vs. non-Aboriginal)	**8.91 (2.87 – 27.68)**	**7.50 (2.34 – 24.05)**	**5.73 (1.15 – 28.49)**	**4.56 (1.04 – 20.02)**
Sibling history of asthma	**2.87 (1.02 – 8.03)**	**3.20 (1.08 – 9.50)**	1.64 (0.41 – 6.53)	-
Maternal non-completion of high school	2.26 (0.97 – 5.26)	1.72 (0.71 – 4.19)	-	-
Paternal non-completion of high school	**3.16 (1.35 – 7.40)**	**3.02 (1.23 – 7.44)**	1.96 (0.55 – 7.06)	-
Household crowding	**6.23 (2.48 – 16.61)**	**4.91 (1.97 – 12.25)**	**3.57 (1.35 – 9.46)**	**5.09 (1.45 – 17.91)**
Interaction term - FokI genotype x paternal ethnicity	7.71 (0.90 – 66.14)	-	-	-

Odds ratios in bold were significant at $P < 0.05$; those underlined were significant at $P < 0.10$.

[1] Odds ratios in models that controlled for multiple host and household socioeconomic factors (age, weight, gender, birth order, duration of exclusive breast-feeding, maternal smoking during pregnancy, number of smokers in household, maternal education, paternal education, household crowding index).

[2] Model only includes the five variables that were independently predictive of ALRI. Nagelkerke $R^2 = 0.564$; model correctly predicted 84.1% of case/control outcomes.

Analysis of predictors of vitamin D status

In the analysis of predictors and patterns of vitamin D status, cases and controls were considered together because the mean and distributions were similar. Significant associations were detected between some participant characteristics and 25(OH)D concentration (Table 8). Notably, the 25(OH)D concentration tended to slightly decrease the later in the winter that the measurement was taken (i.e., with greater time passed since the summer), but the number of days from admission until venous sampling for 25(OH)D measurement (median = 2 days) did not correlate with 25(OH)D concentration.

Table 8. Associations between participant characteristics and vitamin D status

Characteristic	Vitamin D Status	
	Mean 25(OH)D or correlation coefficient (Pearson R or Spearman ρ*)	***P* value**
Age	R = - 0.072	0.419
Gender Boys Girls	 75.9 (SD 20.8) 79.9 (SD 25.4)	0.354
Weight	R = - 0.029	0.747
Number of days from January 1	ρ = - 0.213	**0.016**
Number of days between admission and venous sampling for 25(OH)D measurement	ρ = - 0.046	0.717
Maternal Ethnicity Aboriginal European	 84.6 (SD 22.6) 75.3 (SD 19.8)	**0.041**
Paternal Ethnicity Aboriginal European	 78.6 (SD 28.2) 76.3 (SD 19.2)	0.621
Current exclusive breastfeeding Yes No	 62.2 (SD 36.9) 78.1 (SD 20.2)	**0.029**
Duration of exclusive breastfeeding	ρ = - 0.095	0.455
Weight-adjusted vitamin D intake (mcg/day/kg)	ρ = 0.319	**0.012**
Current infant formula intake Yes No	 83.1 (SD 23.7) 73.2 /(SD 17.8)	**0.008**
Current milk intake (cow or soy) Yes No	 74.3 (SD 14.9) 81.0 (SD 26.3)	0.079

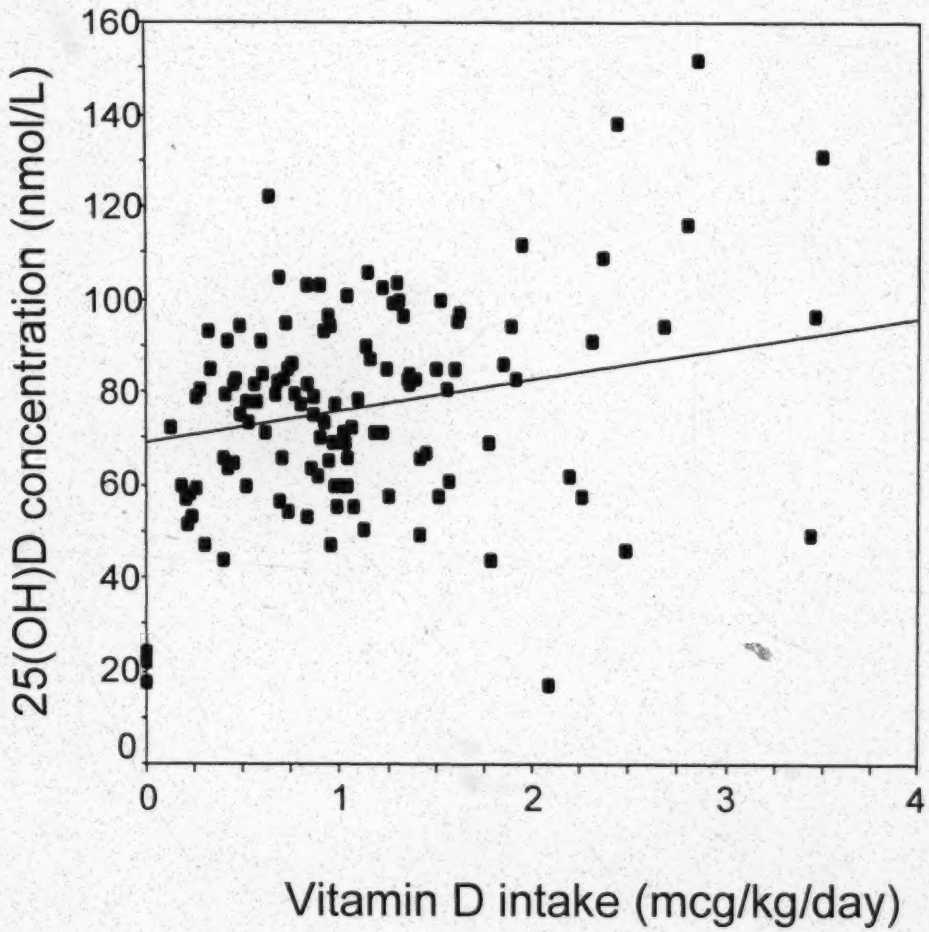
Characteristic	Vitamin D Status	
	Mean 25(OH)D or correlation coefficient (Pearson R or Spearman ρ*)	***P* value**
Current vitamin D supplement (not including infant formula)		**0.004**
Yes	86.7 (SD 25.5)	
No	74.3 (SD 18.9)	
Location of child's residence		
Edmonton and northward	78.7 (SD 21.9)	0.076
South of Edmonton	69.2 (SD 22.8)	
Asthma or eczema		
Yes	79.7 (SD 23.7)	0.925
No	79.2 (SD 24.6)	
Maternal education		
High School Complete	78.4 (SD 23.5)	0.523
High School Incomplete	81.7 (SD 23.5)	
Paternal education		
High School Complete	80.7 (SD 23.8)	0.256
High School Incomplete	75.0 (SD 26.7)	

*Pearson R was calculated for predictor variables that were normally distributed; Spearman ρ was calculated for variables that were not normally distributed.

However, in a multiple linear regression model, weight-adjusted vitamin D intake ($\beta = 0.322$, $P < 0.001$) was the only variable that remained independently associated with vitamin D status, although the correlation was weak (Figure 1).

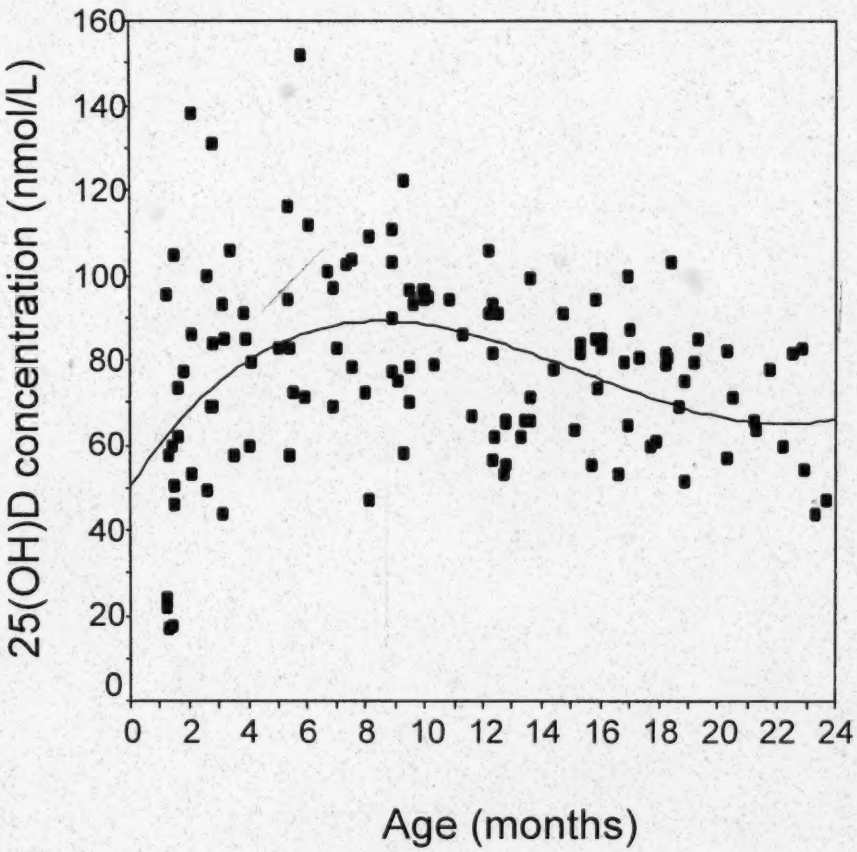
The median vitamin D intake in the first year of life was 9.1 mcg/day (IQR 5.9) and 7.8 mcg/day (IQR 6.9) in the second year of life. Only 41.8 % (28/67) infants (< 1 year of age) met the recommended Health Canada intake of 10 mcg/day (400 IU/day), yet 86.6 % (58/67) had an intake of at least 5 mcg/day (200 IU). Among children one year and older, 76.3 % (45/59) met the recommended adequate intake (AI) of 5 mcg/day (200 IU/day). Of the 23 children who did not meet the AI of 5 mcg/day, 19 (82.6 %) had VDI-I, yet even among the 103 children who did meet the AI, 51 (49.5 %) had VDI-I.

Figure 1. Association between 25(OH)D concentration and weight-adjusted vitamin D intake. The fit line represents a weak positive linear relationship ($R^2 = 0.110$, $P < 0.001$). The figure excludes one outlier with vitamin D intake > 10 mcg/kg/day.



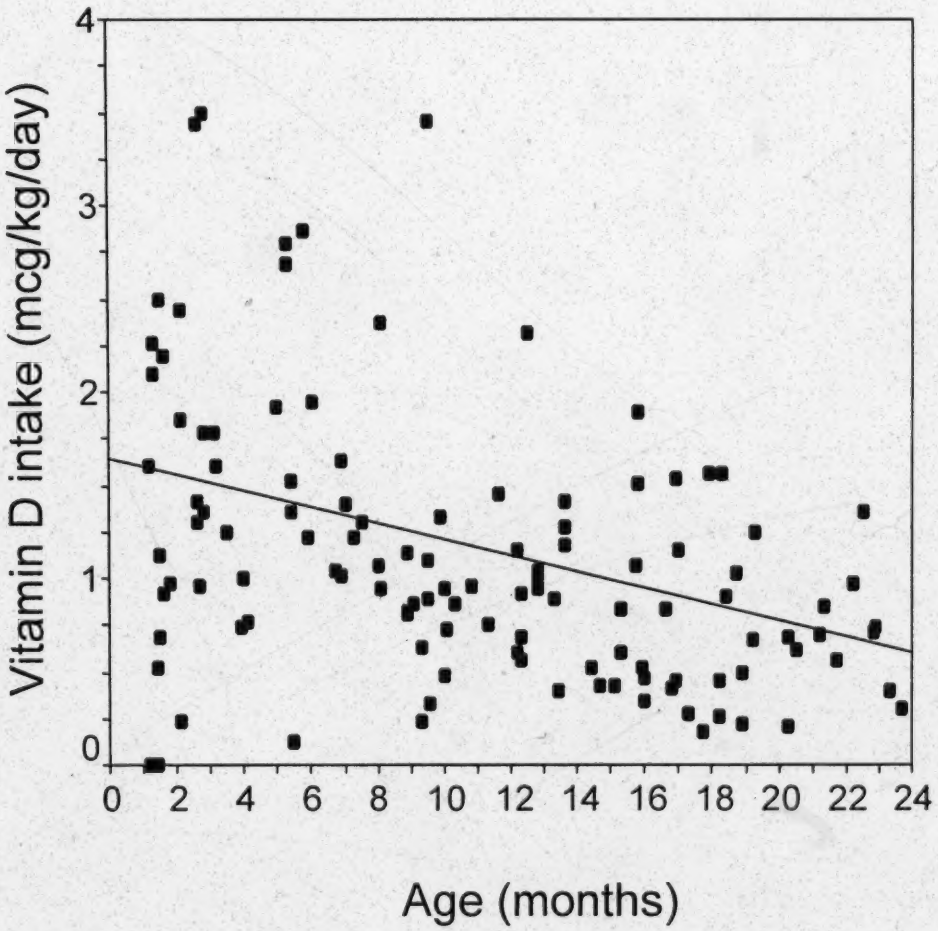
Although age was not linearly associated with 25(OH)D concentration, a cubic regression model fit the data weakly but significantly (Figure 2).

Figure 2. Association of age and 25(OH)D concentration. The fit curve is based on a cubic regression model that weakly fit the data: [25(OH)D] = 50.28 + 10.49*(age) – 0.85*(age)^2 + 0.02*(age)^3, for which $R^2 = 0.165$, $P < 0.001$. The mean 25(OH)D concentration was significantly different among four post-hoc age subgroups: 74.2 nmol/L for the group aged < 6 months, 88.6 nmol/L for ages 6 to 12 months, 75.5 nmol/L for ages 12 to 18 months, and 70.0 for the group > 18 months of age ($P = 0.009$).



Although the predictive capacity of the model was poor, the scatterplot of the relationship shown in figure 2 highlights the apparent tendency for vitamin D concentrations to be highest in the second half of the first year of life. Also apparent was the lack of very high 25(OH)D measurements (> 100 nmol/L) in the group of participants over the age of one year (3/59) compared to those in the first year of life (15/70). Consistent with the trend towards lower 25(OH)D concentrations with increasing age, there was also a tendency for weight-adjusted vitamin D intake to decrease with age (Figure 3).

Figure 3. Association of weight-adjusted vitamin D intake and age. A negative linear regression line weakly fit the data ($R^2 = 0.074$, $P = 0.002$). The mean weight-adjusted vitamin D intake was significantly different among four post-hoc age subgroups: 1.48 mcg/kg/day for the group aged < 6 months, 1.45 mcg/kg/day for ages 6 to 12 months, 0.89 mcg/kg/day for ages 12 to 18 months, and 0.72 mcg/kg/day for the group > 18 months of age ($P = 0.011$).



As observed in the scatterplots (Figures 1 and 2), there were 3 high vitamin D outliers [25(OH)D > +2 SD above the mean: > 125 nmol/L]. All of the high outliers had a large exogenous intake of dietary vitamin D3: two (152 and 131 nmol/L) were formula-fed infants also receiving a daily vitamin D supplement (total daily vitamin D intake of 2.86 mcg/kg and 3.49 mcg/kg) and the third (138 nmol/L) was a two month-old breastfed infant receiving a daily vitamin D supplement (2.44 mcg/kg/day).

There were 4 low vitamin D outliers [25(OH)D < - 2 SD below the mean: < 29 nmol/L]. Three low outliers were the only three exclusively breastfed infants not receiving vitamin D supplementation in the study. In contrast, the other 7 of a total of 10 exclusively breastfed infants in the study were receiving daily vitamin D supplementation (mean 6.9 mcg/day, range of 2.9 to 10 mcg/day) and had a mean 25(OH)D concentration of 79.9 nmol/L (range of 46.1 to 138.0 nmol/L). However, the fourth low outlier was a 7-week old infant who was a control participant (elective surgery for inguinal hernia repair) without known risk factors for malabsorption. He had a 25(OH)D concentration of 17 nmol/L (by both RIA and LC/MS) despite being formula-fed since week two of life and thus receiving a substantial amount of vitamin D (2.09 mcg/kg/day).

CHAPTER FOUR

Vitamin D Status, Vitamin D Receptor Polymorphisms and the Severity of Acute Lower Respiratory Tract Infection in Hospitalized Children

Objective

To explore potential associations between vitamin D status or vitamin D receptor polymorphisms and the severity of acute lower respiratory tract infection among infants and young children between 1 and 24 months of age admitted to a tertiary care pediatric centre in Edmonton.

Hypothesis

We postulated that among admitted patients with ALRI, lower serum concentrations of 25(OH)D and the T and/or f alleles would be predictors of greater illness severity.

Methods

Study design

This was a prospective cohort study involving the case participants included in the case-control study described in Chapter Two. Because sample size calculations were based on the case-control study, this cohort study was an exploratory pilot study that was not deliberately powered to detect statistically significant associations involving the primary outcome measures.

Study Setting

Participants were recruited at the Stollery Children's Hospital in the Walter C. Mackenzie Health Sciences Centre, Edmonton, Alberta (latitude 53°N).

Study Duration

Participants were recruited during two winter seasons: January 1 – March 31, 2005 and January 1, 2006 – March 31, 2006, as described in Chapter Two.

Participants

Participants were patients between the ages of 1 month (30 days) and 2 years (up to the day prior to second birthday) at the time of admission to the Stollery Children's Hospital general pediatric inpatient ward or pediatric intensive care unit (PICU) with the admitting diagnosis of an acute lower respiratory tract infection (ALRI), including uncomplicated bronchiolitis, pneumonia, or an acute lower respiratory tract infection fulfilling at least one of the case definitions described below ('case definitions'). The inclusion criteria, exclusion criteria, and recruitment process were described in Chapter Two.

Data Collection and Analysis

Outcome Measures

The *a priori* primary measure of disease severity was the duration of supplemental oxygen therapy (DOSOT), from time zero defined when the patient was initially assessed by emergency health care personnel until either the time that oxygen therapy was first discontinued (preceding 24 hours free from supplemental oxygen), or until hospital discharge if oxygen therapy was discontinued at discharge. The secondary outcome measure was the length of hospital stay (LOS), from the time of presentation to medical attention until the time of discharge recorded in the hospital database. Sensitivity analyses were conducted using alternative measures of DOSOT and LOS whereby time zero was defined as the time of inpatient admission. An additional secondary measure of disease severity was the oxygen saturation by pulse oximetry (on room air) when first measured by health care personnel, analysed as a continuous variable and as a dichotomous measure of the presence or absence of hypoxemia (oxygen saturation < 90%). Additional participant characteristics were also considered as potential risk factors for ALRI severity.

Data Collection Instruments and Assays

Data was collected by a parent/caregiver questionnaire, food frequency questionnaire, and weight measurement as described in Chapter Two. Vitamin D status (25-hydroxyvitamin D concentration) and VDR genotypes were determined as outlined in Chapter Two.

The following clinical parameters were collected by hospital chart review and recorded on a *Clinical Data Collection Form* (Appendix G):

- date and time of admission and discharge were extracted from the hospital database.
- date and time of emergency department (ED) assessment were manually extracted from hospital records; in cases where the patient was initially assessed at a location other than the Children's hospital, the ED date/time were those of presentation to the community hospital or health centre.

- evidence of respiratory distress assessed during the nurse or physician physical exam in the ED
- oxygen saturation by pulse oximetry when first assessed on room air (by emergency medical personnel or in the ED)
- highest body temperature recorded in the first 24 hours of admission including the temperature recorded upon presentation to the ED
- the date and time of the first note of 'RA' (room air) preceding 24 hours free from supplemental oxygen, or lasting until discharge.
- illness duration prior to admission, defined as the number of days from the first day of illness, prior to which the child was completely 'well' (e.g., feeding normally, normal energy, no cough, no fever, no respiratory distress) until and including the day of admission.
- administration of corticosteroids or antibiotics for acute treatment in the ED or ward.
- nasal swab/aspirate results for virological studies
- chest x-ray report (if an x-ray was performed)

Statistical Analysis

Non-parametric tests were applied for the comparison of non-normally-distributed disease characteristics among groups of interest, including the Mann-Whitney U rank test for comparisons of continuous variables between two groups and the Kruskal-Wallis test for comparison of continuous variables among three groups. Spearman rank correlation (ρ) was used to assess the significance of bivariate associations between two continuous variables. Associations between pairs of categorical variables were analysed by Chi-square or Fisher's exact tests.

Kaplan-Meier survival curves were plotted to assess the DOSOT of various groups of interest (as defined by potential disease severity risk factors). Censored cases were those participants who were transferred to another hospital with a persistent supplemental oxygen requirement, and thus for whom the actual duration of oxygen needs or duration of hospitalization was unknown. Survival curves among were compared by the log-rank test, or Breslow test if the proportional hazards assumption was not supported (e.g., crossing of the survival curves). Cox proportional hazards regression analysis was used to calculate hazard ratios (HR) associated with disease severity risk factors. Time-dependent covariates were calculated to assess adherence to the assumption of proportional hazards, whereby a statistically-significant hazards ratio associated with a time-dependent covariate indicated that the proportionality assumption was not supported.

All reported confidence intervals (CI) were at 95% and all reported *P* values were two-sided; $P < 0.05$ was considered to be statistically significant. In the data tables, *P* values < 0.05 are highlighted in bold, and *P* values 0.05 – 0.10 are underlined. Statistical analyses were conducted using SPSS v.10.0 (SPSS Corporation, Chicago, IL).

Results

There were 64 case participants with 25(OH)D levels, among whom follow-up data regarding duration of supplemental oxygen therapy (DOSOT) was available for 61 (95.3 %). Three cases were censored due to incomplete follow-up data (i.e., these patients were transferred to another hospital while still receiving supplemental oxygen therapy). Since the primary measure of disease severity was not normally distributed (Kolmogorov-Smirnov Z = 1.370, $P = 0.047$), differences in DOSOT between groups of interest were compared using nonparametric tests. Because there were few censored cases that could be included in the analysis (for 2/3 censored cases, data was not available regarding the time that oxygen therapy was initiated), initial nonparametric testing excluded cases for which follow-up was incomplete. Survival analysis, incorporating the censored cases, was applied to confirm the findings and for purposes of graphical representation.

Association of vitamin D status and ALRI severity

There were no significant associations between 25(OH)D concentration and the DOSOT from the time of initial presentation (Spearman $\rho = -0.184$, $P = 0.156$), the DOSOT from the time of inpatient admission (Spearman $\rho = -0.187$, $P = 0.140$), the length of stay (LOS) from the time of initial presentation (Spearman $\rho = -0.129$, $P = 0.321$), the LOS from the time of inpatient admission (Spearman $\rho = -0.160$, $P = 0.207$), or the initial oxygen saturation by pulse oximetry (Spearman $\rho = 0.042$, $P = 0.755$).

There was a trend towards a longer median duration of hospitalization and supplemental oxygen therapy among participants with VDI compared to those with ideal vitamin D status, but none of the differences severity reached statistical significance (Table 9).

Table 9. ALRI characteristics and markers of severity among hospitalized participants, subgrouped according to vitamin D status (N = 64).

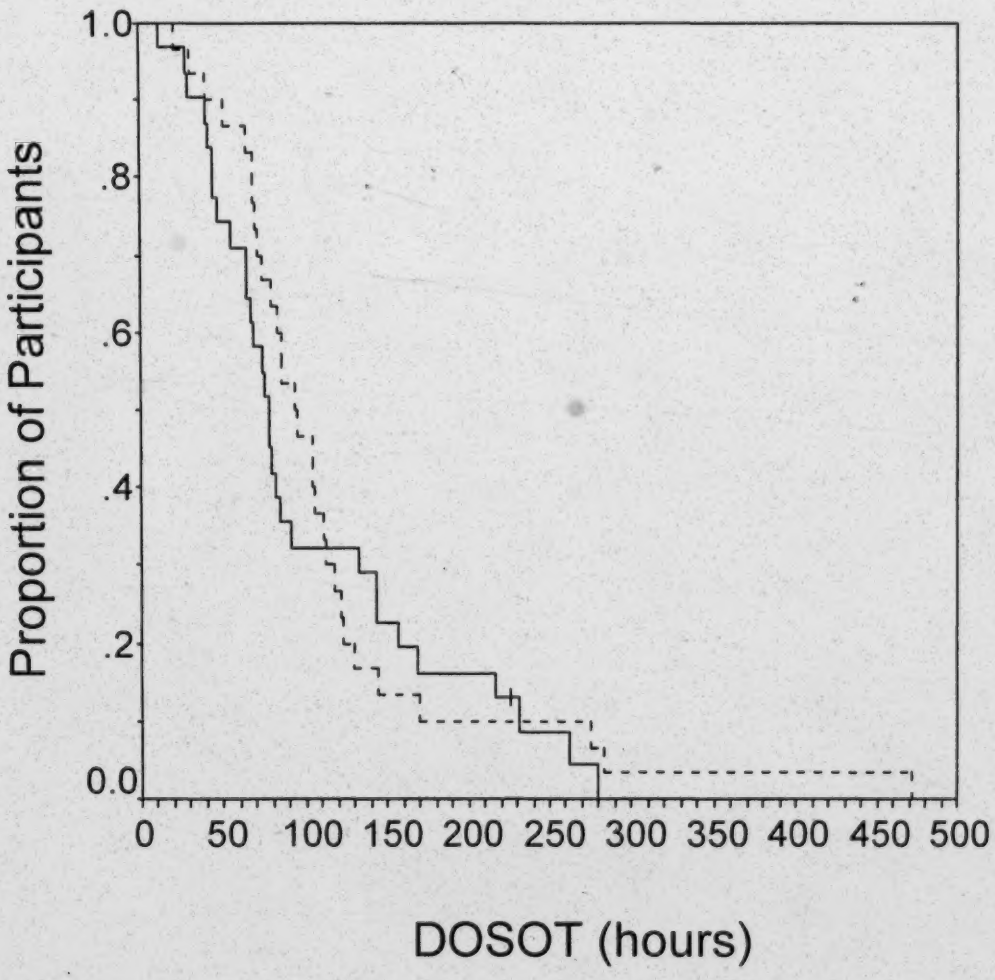
Characteristic	N	Vitamin D Status (nmol/l)		*P* value
		[25(OH)D] < 80	[25(OH)D] ≥ 80	
Mean age (months)	64	7.8 (SD 6.6)	9.0 (SD 5.3)	0.431
ALRI type				
Bronchiolitis	60	31 (93.9 %)	29 (93.5 %)	1.000
Pneumonia	4	2 (6.1 %)	2 (6.5 %)	
RSV-positive	64	27 (81.8 %)	25 (80.6 %)	1.000
Median number of days unwell prior to presentation	56	4.5 (IQR 4.0)	5.0 (IQR 3.0)	0.956

Characteristic	N	Vitamin D Status (nmol/l)		*P* value
		[25(OH)D] < 80	[25(OH)D] ≥ 80	
Median duration of supplemental oxygen therapy from initial presentation (hours)	60	93.5 (IQR 53.6)	73.8 (IQR 95.5)	0.204
Median duration of supplemental oxygen therapy from time of ward admission (hours)	61	91.2 (IQR 50.7)	67.6 (IQR 93.6)	0.149
Median length of stay in hospital from initial presentation (hours)	60	113.5 (IQR 57.7)	97.0 (IQR 91.3)	0.408
Median length of stay in hospital from time of ward admission (hours)	61	111.8 (IQR 56.2)	88.4 (IQR 87.8)	0.267
Median Oxygen saturation at presentation (%)	59	89.0 (IQR 8.3)	90.0 (IQR 5.0)	0.778
Hypoxemia upon initial presentation	59	16 (53.3 %)	12 (41.4 %)	0.438
Administered an antibiotic	64	11 (33.3 %)	10 (32.3 %)	1.000
Administered a steroid	64	4 (12.1 %)	9 (29.0 %)	0.229
Admitted to PICU	64	4 (12.1 %)	0	0.114

[1] Analyses of longitudinal outcomes (DOSOT and LOS) exclude 3 censored cases (transferred out before supplemental oxygen needs had resolved). Number of patients in each group: 25(OH) < 80 – 31, 25(OH)D ≥ 80 – 30.

Comparison of Kaplan-Meier survival curves confirmed the lack of a significant association between the primary marker of disease severity (DOSOT from the time of initial presentation) and VDI (Figure 4).

Figure 4. Kaplan-Meier curves demonstrating the duration of supplemental oxygen therapy (DOSOT) from the time of initial presentation among participants grouped according to vitamin D status: VDI ——, vitamin D sufficient - - -; + indicates censored case. The two curves were similar (Breslow statistic 1.10, $P = 0.295$).



There was a trend towards a lower mean 25(OH)D concentration among patients who presented with hypoxemia compared to those who were normoxemic, but the difference was not statistically significant [72.8 nmol/L (SD 23.3) versus 81.1 nmol/L (SD 25.9), $P = 0.200$].

Associations of VDR polymorphisms and ALRI severity

The only significant difference among groups of participants classified by VDR genotype was in the duration of time that the child was considered unwell by the parent/caregiver prior to admission to hospital (Table 10). There was a non-significant trend towards a longer DOSOT and LOS among the patients with TaqI tt genotype, yet this was based on only four patients. As well, there was a trend towards a higher prevalence of hypoxemia among patients with the FokI ff genotype, compared to those with FF or Ff genotypes (Table 10). In an analysis in which the FF and Ff groups were collapsed into one group, patients with the ff genotype were more likely to present with hypoxemia, a difference that was of borderline significance (ff, 66.7 % versus non-ff, 35.0 %; $P = 0.094$). When this analysis was limited to those patients with VDI-I (N = 27), the association of the ff genotype with hypoxemia was strongly significant (ff, 100 % versus non-ff, 38.1 %; $P = 0.016$). In contrast, among those patients with optimal vitamin D status (N = 25), the FokI polymorphism was not associated with an increased risk of hypoxemia (ff, 33.3 % versus non-ff, 31.6 %; $P = 1.000$).

Table 10. ALRI characteristics and markers of severity among hospitalized participants, subgrouped according to vitamin D receptor genotype (N = 56)

Characteristic	VDR FokI Genotype				VDR FokI Genotype			
	FF	Ff	ff	*P* value	TT	Tt	tt	*P* value
Mean age (months)	7.8 (SD 6.5)	7.9 (SD 5.7)	10.2 (SD 5.4)	0.464	6.9 (SD 5.3)	9.7 (SD 6.2)	7.9 (SD 4.0)	0.226
ALRI type								
Bronchiolitis	14 (100 %)	26 (89.7%)	12 (92.3%)	0.465	22 (91.7%)	26 (92.6%)	4 (100%)	0.836
Pneumonia	0	3 (10.3 %)	1 (7.7 %)		2 (8.3 %)	2 (7.1 %)	0	
RSV-positive	11 (78.6%)	26 (89.7%)	9 (69.2 %)	0.180	19 (79.2%)	23 (82.1%)	4 (100%)	0.985
Median number of days unwell prior to presentation	3.0 (IQR 3.0)	5.0 (IQR 3.0)	4.5 (IQR 3.3)	**0.042**	6.0 (IQR 3.0)	4.0 (IQR 2.0)	3.0 (IQR 3.5)	**0.023**
Median duration of supplemental oxygen therapy from initial presentation (hours)*	73.9 (IQR 79.6)	84.0 (IQR 64.0)	84.4 (IQR 68.6)	0.855	92.2 (IQR 57.4)	73.8 (IQR 72.8)	119.4 (IQR 143.0)	0.255

Characteristic	VDR FokI Genotype				VDR FokI Genotype			
	FF	Ff	ff	*P* value	TT	Tt	tt	*P* value
Median duration of supplemental oxygen therapy from time of ward admission (hours)*	71.3 (IQR 79.6)	79.3 (IQR 82.5)	76.3 (IQR 62.1)	0.707	87.7 (IQR 62.8)	68.2 (IQR 64.0)	112.6 (IQR 143.6)	0.564
Median length of stay in hospital from initial presentation (hours)*	109.9 (IQR 115.2)	108.7 (IQR 74.3)	116.6 (IQR 75.8)	0.867	112.0 (IQR 64.4)	108.0 (IQR 73.4)	168.2 (IQR 184.8)	0.624
Median length of stay in hospital from time of ward admission (hours)*	107.7 (IQR 111.3)	102.1 (IQR 75.2)	111.7 (IQR 73.9)	0.677	107.2 (IQR 75.6)	101.1 (IQR 72.8)	164.1 (IQR 182.5)	0.722
Median Oxygen saturation at presentation (%)	91.0 (IQR 8.3)	91.5 (IQR 5.5)	88.0 (IQR 4.5)	0.106	90.5 (IQR 7.5)	90.5 (IQR 5.3)	93.5 (IQR 8.3)	0.347
Hypoxemia upon initial presentation	5 (38.5 %)	9 (33.3 %)	8 (66.7 %)	0.143	10 (47.6%)	11 (40.7%)	1 (25.0 %)	0.684
Administered an antibiotic	5 (35.7 %)	8 (27.6 %)	6 (46.2 %)	0.495	7 (29.2 %)	12 (42.9%)	0	0.193
Administered a steroid	3 (21.4 %)	7 (24.1 %)	2 (15.4 %)	0.827	6 (25.0 %)	5 (17.9 %)	1 (25.0 %)	0.675
Admitted to PICU	1 (7.1 %)	1 (3.4 %)	1 (7.7 %)	0.804	1 (4.2 %)	2 (7.1 %)	0	0.791

*Analyses involving longitudinal measures of severity (DOSOT and LOS) excluded 3 censored cases (transferred out before supplemental oxygen needs had resolved). Number of participants in each group: FF – 12, Ff – 26, ff – 12; TT – 20, Tt – 26, tt – 4.

Comparison of Kaplan-Meier survival curves confirmed the lack of a significant association between the primary measure of disease severity (DOSOT from the time of initial presentation) and either VDR FokI polymorphisms (Figure 5) or VDR TaqI polymorphisms (Figure 6).

Figure 5. Kaplan-Meier curve demonstrating the duration of supplemental oxygen therapy (DOSOT) from the time of initial presentation among participants grouped according to FokI genotype. FF ——, Ff - - -, ff – – –; + indicates censored case. The curves for all three groups were similar (Breslow statistic 0.50, $P = 0.779$).

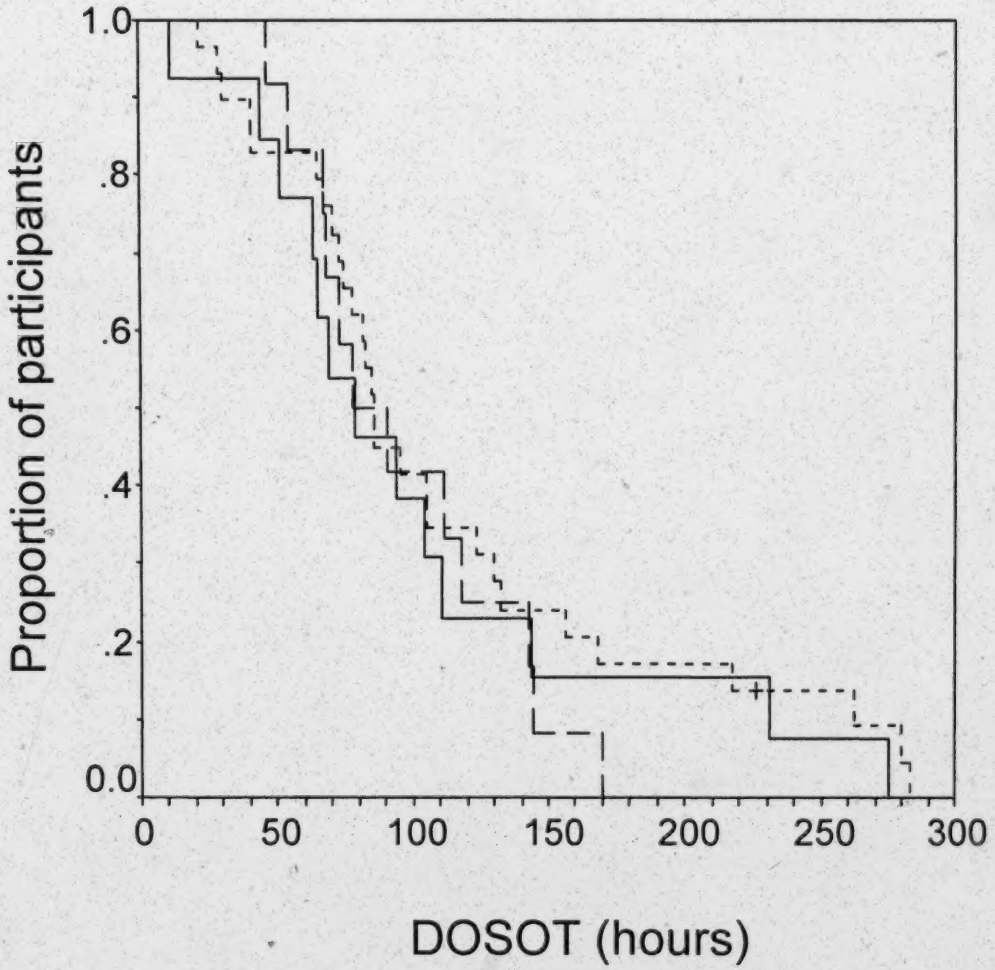
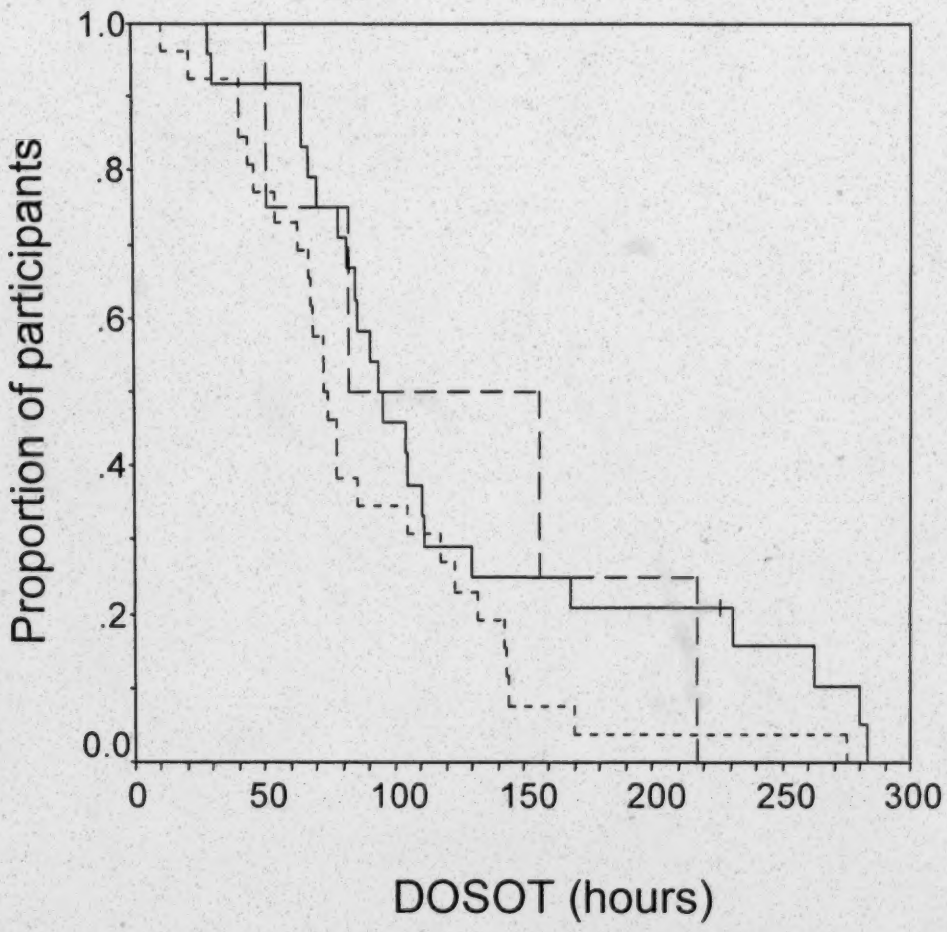


Figure 6. Kaplan-Meier curve demonstrating the duration of supplemental oxygen therapy (DOSOT) from the time of initial presentation among participants grouped according to TaqI genotype. TT ——, Tt - - -, tt – – –; + indicates censored case. The curves for all three groups were similar (Breslow statistic 3.35, $P = 0.188$).



Analysis of additional predictors of ALRI severity

Of multiple participant characteristics considered as potential predictors of disease severity, bivariate analysis revealed that associations of DOSOT with birthweight and birth order were statistically significant (Table 11). Notably, oxygen saturation by pulse oximetry at initial presentation did not correlate with either DOSOT (Table 11) or LOS ($\rho = 0.082$, $P = 0.539$).

Table 11. Association between markers of ALRI severity and characteristics of hospitalized participants (N = 61).

Characteristic	Median duration of supplemental oxygen therapy (hours) or Spearman correlation (ρ)	*P* value
Age	ρ = - 0.131	0.320
Gender Boys Girls	 85.7 (IQR 84.7) 86.2 (IQR 62.9)	0.863
Weight (kg)	ρ = - 0.133	0.314
Year of Recruitment Year 1 (2005) Year 2 (2006)	 78.8 (IQR 66.8) 76.1 (IQR 60.1)	0.712
Percent oxygen saturation at initial presentation	ρ = - 0.018	0.896
Number of ALRIs requiring overnight hospitalization	ρ = 0.192	0.141
Previous Asthma diagnosis Yes No	 67.1 (IQR 167.4) 82.3 (IQR 65.1)	0.459
Previous Eczema diagnosis Yes No	 59.7 (IQR 95.4) 85.7 (IQR 68.9)	0.171
Number of episodes of 'ear infections'	ρ = - 0.100	0.445
Gestational age at birth (weeks)	ρ = - 0.012	0.930
Birthweight (grams)	ρ = 0.266	**0.040**
Immunizations are up-to-date for age Yes No	 81.9 (IQR 72.9) 91.5 (IQR 126.7)	0.440
Current exclusive breastfeeding Yes No	 85.7 (IQR 210.2) 82.3 (IQR 66.7)	0.639
Breastfeeding index (1 = 6 months, or to present if age < 6 months)	ρ = - 0.112	0.393
Birth order	ρ = 0.462	**< 0.001**
Maternal smoking during pregnancy* Yes No	 100.0 (IQR 84.2) 78.4 (IQR 57.4)	0.217
Regular daycare attendance* Yes No	 73.4 (IQR 88.0) 84.0 (IQR	0.132

Characteristic	Median duration of supplemental oxygen therapy (hours) or Spearman correlation (ρ)	*P* value
'Sick contact' in the week prior to hospitalization		
Yes	82.3 (IQR 59.7)	0.824
No	85.8 (IQR 91.2)	
Number of smokers in household*	ρ = 0.181	0.173
Maternal ancestry		
European/Caucasian	78.1 (IQR 62.4)	0.475
Aboriginal	104.5 (IQR 158.4)	
Other	78.4 (IQR 141.7)	
Paternal ancestry		
European/Caucasian	85.7 (IQR 64.4)	0.368
Aboriginal	90.8 (IQR 166.8)	
Other	67.7 (IQR 43.5)	
Maternal history of asthma*		
Yes	73.4 (IQR 100.0)	0.346
No	88.3 (IQR 62.9)	
Paternal history of asthma *		
Yes	87.9 (IQR 52.9)	0.691
No	83.6 (IQR 75.5)	
Sibling history of asthma*		
Yes	95.2 (IQR 72.8)	0.490
No	78.4 (IQR 58.1)	
Maternal education level*		
High School Complete	81.6 (IQR 56.3)	0.435
High School Incomplete	114.8 (IQR 220.0)	
Paternal education level*		
High School Complete	78.3 (IQR 64.2)	0.358
High School Incomplete	104.9 (IQR 158.4)	
Crowding index* [(No. adults + No. children) / No. sleeping rooms in house]	ρ = 0.022	0.871
Dietary vitamin D intake / body weight (mcg/day/kg)	ρ = - 0.111	0.403

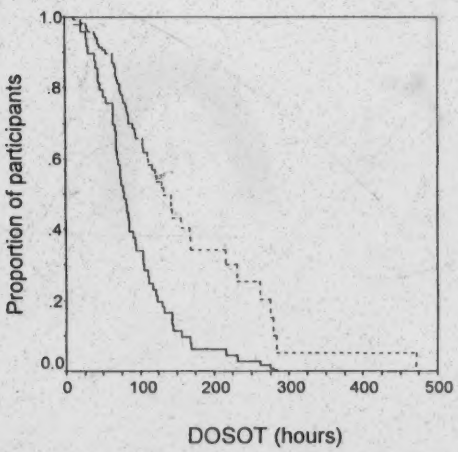
* N = 128 due to missing questionnaire data

Plotting of Kaplan-Meier survival curves and Cox regression analysis confirmed the lack of association between disease severity and all but two of the potential categorical predictor variables, maternal education and birth order (birthweight was not associated with DOSOT in survival analysis). Reduced odds of ending supplemental oxygen requirements was associated with higher birth order (Hazard Ratio 0.600, 95 % CI 0.469 – 0.767; $P < 0.001$) and maternal high school non-completion (Hazard ratio 0.386, 95 % CI 0.187 – 0.797; $P = 0.010$). However, the hazard ratios for both of these variables did not remain constant over

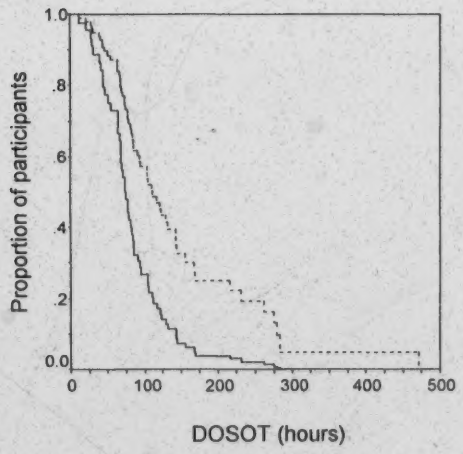
time and thus did not support the proportional hazards assumption, as evidenced graphically as well as by the statistical significance of the hazards ratios for time-dependent covariates (Figure 7); therefore, the risk estimates were interpreted with caution. Because none of the primary variables of interest were significantly associated with disease severity, multivariate Cox proportional hazards modeling was not performed.

Figure 7. Survival curves representing the duration of supplemental oxygen therapy, based on Cox regression analysis for participants grouped according to a) maternal education level and b) birth order (categorized into dichotomous groups). Lines represent the following groups: a) Maternal high school non-completion - - -, Maternal high school completion —— ; b) Third or higher birth order - - -, First or second birth order —— . Hazards ratio for time-dependent covariates [variable*ln(time)] were statistically significant (Maternal education level: HR 1.286, 95 % CI 1.087 – 1.522, $P = 0.003$; Birth order: HR [illegible], 95 % CI 0.740 – 0.945, $P = 0.004$).

a)



b)



CHAPTER FIVE

Discussion and Conclusions

Summary of major findings

In this case-control study of the role of vitamin D in early childhood ALRI susceptibility, 64 infants and young children admitted to hospital with acute lower respiratory tract infection (ALRI) during the winter were compared with 65 control participants (elective surgery patients) with no history of hospital admission for ALRI. Vitamin D status, indicated by serum 25-hydroxyvitamin D concentration, was not associated with the risk of hospitalization for ALRI during the first two years of life in this population. Specifically, the mean 25(OH)D concentrations were very similar among cases and controls, and the prevalence of vitamin D insufficiency (VDI) using two established thresholds (40 and 80 nmol/L) was not significantly different between the case and control groups. Although the cases had a significantly higher mean daily intake of vitamin D than controls, this did not signify that vitamin D intake was a meaningful ALRI risk factor, since the difference became non-significant after adjusting for the younger age of the cases. However, the risk of ALRI was associated with the presence of a function-altering single-nucleotide polymorphism (SNP) in the gene that encodes the vitamin D receptor (VDR). The VDR FokI ff genotype increased odds of ALRI by approximately 6-fold (unadjusted) compared to non-ff genotypes (FF and Ff). There was no association between ALRI susceptibility and another VDR SNP identified by the TaqI restriction enzyme.

To further explore potential clinical associations between vitamin D status and the host response in ALRI, we conducted a pilot cohort study involving the prospectively-recruited case participants. By measuring ALRI severity on the basis of the duration of supplemental therapy or length of stay in hospital, we observed a trend towards reduced ALRI severity among participants with optimal vitamin D status (> 80 nmol/L) compared to those with VDI, but none of the associations reached statistical significance. Neither of the VDR SNPs was associated with ALRI severity. However, there was a trend towards a higher prevalence of hypoxemia at presentation among patients with the FokI ff genotype, an association that became significant when the analysis was limited to patients with suboptimal vitamin D status (VDI-I).

Lack of association between vitamin D status and the risk or severity of ALRI

To our knowledge, this is the first study to assess the potential association between vitamin D status and childhood ALRI susceptibility and severity in a northern, developed country. Because of the lack of precedent data, the primary hypothesis for this exploratory study was necessarily two-sided. On one hand, there has long been evidence of an association between lower respiratory tract disease and rickets[223], suggesting that milder states of vitamin D deficiency may also predispose to ALRI. More recently, there has been accumulating data

revealing vitamin D to be a potent modulator of systemic and local inflammatory processes[171], suggesting its potential beneficial role in limiting viral infection or dampening the immunopathogenic cascades that cause the clinical manifestations of bronchiolitis, the most common ALRI in young Canadian children. In northern Canada, there are extraordinarily high rates of severe bronchiolitis[51], a largely unexplained phenomenon that may coincide with a high prevalence of VDI among northern infants[122,224], suggesting a potential nutritional target for ALRI prevention. Data demonstrating a high wintertime prevalence of VDI among older children and adolescents in the general population[120] suggested that VDI may likewise be common among young children, for which Canadian data were previously unavailable.

Using RSV bronchiolitis as a model because it is the best-studied viral ALRI in childhood, there are several candidate mechanisms by which activated vitamin D could exert an anti-viral or anti-inflammatory role that would limit RSV-induced lung damage. For example, the active metabolite of vitamin D, 1,25(OH)2D, was found to downregulate the expression of TLR4[192], one of the main cellular receptors to which RSV binds[9]. The peak of clinical disease severity coincides with T cell proliferation[9], which is suppressed when vitamin D is added to human peripheral blood mononuclear cell lines[172]. Vitamin D also suppresses TNFα synthesis in monocytes via inhibition of NFκB/relΛ translocation to the nucleus[192], and may lower serum concentrations of MMP-9[201], one of the mediators of RSV-induced tissue damage. Furthermore, the synthesis of chemokines implicated in the RSV-induced inflammatory response (notably CCR5 and MIP-2) were significantly suppressed by the administration of vitamin D3 analogues in mice[176].

On the other hand, several studies have suggested that vitamin D skews the immune system towards a Th2-predominant response. Because Th2 cells are postulated to be implicated in a greater severity of RSV bronchiolitis[9], it was possible that VDI would be paradoxically found to be protective against severe viral ALRI. However, this hypothesis was not supported by asthma animal models that have shown that despite the upregulation of certain Th2 cytokines by activated vitamin D administration, the effect on pulmonary cytology and histology was actually favourable[185]. However, some evidence of cross-talk between vitamin D and inflammatory cascades suggests a possible pro-inflammatory role. For example, vitamin D may upregulate CX3CR1 chemokine receptor[176] and may downregulate the expression of PPARγ[239], the binding of which inhibits the synthesis and release of several proinflammatory chemical mediators from RSV-infected cells[29] and reduces MMP-9 secretion by bronchial epithelial cells[31]. As indirect support for this hypothesis, breastfed infants generally have poorer vitamin D status than formula-fed infants[240] but may be at lower risk of bronchiolitis[45].

The present data indicate that in this population of children aged 1 month to 2 years living primarily in Edmonton and its surrounding areas, systemic vitamin D status did not affect the risk or severity of ALRI in either of the directions suggested by the two opposing hypotheses. This suggests that either vitamin D is not a clinically important player in the immune response again viral respiratory pathogens, or alternatively, that the degree to which vitamin D is active in the host response is not directly related to the quantity of bodily vitamin D stores as reflected by the 25(OH)D serum concentration. The lack of difference in means was unlikely to be due to a false-negative (type II) error because the study was

highly powered to detect a small between-group difference in mean 25(OH)D concentration (10 nmol/L). Furthermore, the finding was reinforced by a subgroup analysis (not planned a priori) in which there was no difference between controls and the subgroup of cases with severe disease (defined as either hypoxemia on presentation or supplemental oxygen needs beyond three days). However, because the 25(OH)D levels were generally higher than predicted, the study was not powered to detect reasonable differences in the risk of ALRI associated with VDI-C (< 40 nmol/L), as planned. Even though the prevalence of VDI-C was higher among cases, the very small number of participants in this group precluded drawing any conclusions. Using the less conservative VDI threshold (< 80 nmol/L), no significant differences were apparent between the groups, and in fact the prevalence of VDI-I in the control group was slightly higher than in the case group (56.9 % versus 51.6 %).

The only previously published study addressing a direct link between vitamin D status and ALRI found that sub-continental Indian children with severe ALRI were at significantly higher risk of VDI than healthy controls[225]. Although details regarding the clinical manifestations or etiology of the ALRIs were not described, a substantial proportion of the children were more likely to have had bacterial pneumonia than bronchiolitis, given the inclusion of children up to the age of 5 years and the developing country setting[241]. In contrast, the cases in the present study were younger than 2 years and virtually all met the clinical case definition for bronchiolitis (88 % had a laboratory-confirmed viral infection).

Differences in predominant ALRI etiology may be a possible explanation for the divergent results between the two studies, yet it is also possible that the effect of VDI on the host response in ALRI is only clinically perceptible in the context of at least a moderate deficiency state (i.e., < 50 nmol/L, the threshold used in the Indian study). Although direct comparison may be limited by inter-laboratory variations, the vitamin D status of the Indian children was generally much poorer than the Canadian children [mean 25(OH)D concentration in control participants: Indian - 34.8 nmol/L versus Canada - 77.2 nmol/L]. Thus, in the present study population in which so few participants had VDI-C, any potential effects of a sub-threshold vitamin D status may not have been apparent.

The data regarding ALRI severity generally corroborated the lack of an independent association with vitamin D status found in the case-control analysis. However, the pilot cohort study was only designed to be exploratory, and thus may have been underpowered to show that the trends towards a longer duration of supplemental oxygen therapy and length of hospital stay in patients with VDI-I were statistically significant.

Significant association of the VDR FokI single-nucleotide polymorphism (SNP) and the risk of ALRI

Despite the negative findings with respect to case-control differences in 25(OH)D concentrations, the significant association of the FokI ff genotype with ALRI susceptibility, and possibly with the severity of disease at presentation, provided evidence that vitamin D-related biochemical pathways are involved in the host response in childhood ALRI. This is the first report of an association between a VDR SNP and an infectious respiratory disease in childhood. Although it is possible that the observed clinical differences were related to a

polymorphism in a neighbouring gene to which the FokI polymorphic site is strongly linked or this was a false-positive finding, the association of the FokI ff genotype with ALRI susceptibility is very consistent with published laboratory and clinical data.

The VDR encoded by the f allele has been shown to be less active relative to the more common and structurally-distinct F variant, likely because the C → T substitution in the 5' translation initiation site of the f allele creates a protein that is three amino acids longer and less readily binds to the vitamin D response elements in target genes[162]. Clinically, the hypofunctional consequences of the ff genotype in children have been most clearly shown in terms of a significantly decreased rate of intestinal calcium absorption and reduced bone mineral density (BMD)[242,243]. In some adult studies, the FokI ff genotype has been associated with an increased risk of extra-skeletal diseases, including reduced insulin secretion[244], severe diabetic retinopathy[245], and colorectal carcinoma[246]. Adult data have also shown that the VDR f allele is adversely associated with tuberculosis susceptibility[211] and treatment response[169]. Furthermore, laboratory data revealed that the f allele is less effective in mediating the suppression of lymphoproliferation by vitamin D[226]. In the context of a viral ALRI, extrapolation of these findings suggests that the expression of the f allele, or perhaps the absence of the F allele, could predispose to reduced vitamin D-mediated anti-viral effects and poorer counter-regulation of inflammatory cascades, potentially leading to more severe disease (i.e., the need for hospitalization). Because bronchiolitis severity may be related to atopic disposition, it is interesting to note that two recent studies found associations of asthma susceptibility with several VDR SNPs including the TaqI SNP, but not with the FokI SNP[247,248].

The association of ALRI susceptibility with the FokI SNP in the absence of an observable independent effect of 25(OH)D concentration raised the prospect of a gene-environment interaction. In fact, the FokI ff genotype only appeared to significantly increase the risk of ALRI among those children with VDI (using 80 nmol/L as the threshold), whereas the effect was weaker and non-significant among those with optimal vitamin D status. Although this subgroup analysis must be interpreted cautiously because each group included few participants with the ff genotype, the accentuation of the increased risk of ALRI with the ff genotype in children with suboptimal vitamin D status is biologically plausible. In studies of the inhibitory effect of vitamin D on the growth of human peripheral blood mononuclear cells, the proliferation of cells expressing VDRs encoded by the f allele could be maximally inhibited to the same degree as cells with the FF genotype, given an adequate concentration of 1,25(OH)2D[226]. An optimal quantity of stored vitamin D may overcome the relative receptor hypo-activity in children carrying the ff genotype, neutralizing any risk differences once a certain threshold has been surpassed.

One of the major limitations in the use of case-control studies to establish genetic associations with disease outcomes is the possibility that ethnic differences in the study sample bias the findings, a phenomenon referred to as population stratification[249]. In the present study, the FokI ff genotype was more common in children with Aboriginal heritage, an ethnic group in which several ALRI risk factors were over-represented, and was also clustered with other ALRI risk factors including a shorter duration of exclusive breastfeeding, maternal smoking during pregnancy, and an increased number of smokers in the household. This suggested the possibility of an 'innocent bystander effect' whereby the

FokI ff genotype was more commonly carried by Aboriginal children who were at a higher risk of ALRI due primarily to social determinants of health. However, the association between the FokI SNP and ALRI susceptibility remained strong and significant after controlling for ethnicity (adjusted OR = 5.1) or multiple host and household risk factors (adjusted OR = 6.9), and was included in the final logistic regression model. To more convincingly exclude population stratification, the analysis was repeated in the subgroup of participants for whom both parents were of European background and an even stronger association was observed (unadjusted OR = 18.3). Nonetheless, the confidence intervals were quite large on these estimates, and it remains possible that the significant findings resulted from a spuriously low prevalence of the ff genotype in the control sample.

Several other genetic polymorphisms have been reported to be associated with RSV bronchiolitis susceptibility (e.g., IL-4, IL-10 , IL-13 , CX3C chemokine receptor , and CCR5 polymorphisms)[35,36,37,38,39,40], but the strength of the FokI SNP association was much greater than those previously reported, most of the earlier studies included adult control populations, and most did not address population stratification. Also, the implication that the VDR mediates the host response in ALRI may be more clinically relevant than the SNPs in other molecules because the ligand of the VDR is a metabolite of a micronutrient that could readily be administered for ALRI prophylaxis or therapy.

Further elaboration of the effects of the interplay between circulating vitamin D metabolites and the VDR on the host response to ALRI is required before firm conclusions can be drawn about its clinical relevance. Yet the data in the present study do suggest that although 25(OH)D serum concentration is a useful marker of vitamin D stores, a complete understanding of an individual's *functional* vitamin D status requires a measure of VDR responsiveness. An additional component of one's functional vitamin D status, not investigated in the present study, may involve the rate at which circulating 25(OH)D is converted to activated 1,25(OH)2D. Studies in tuberculosis patients have clearly shown that local production of 1,25(OH)2D can occur in type II alveolar macrophages and lymphocytes[218]. Specific cytokines have been found to modulate localized vitamin D metabolism; for example, IFNγ appears to increase the synthesis of 1,25(OH)2D by disrupting negative feedback mechanisms[199]. Considering RSV bronchiolitis, in which disease severity inversely correlates with IFNγ secretion[19], it is possible that one of the factors that accounts for the milder form of illness associated with a Th1 immune responses is the local upregulation of anti-inflammatory 1,25(OH)2D. This hypothesis begs the question of whether serum concentrations of 25(OH)D adequately reflect the extent of autocrine or paracrine vitamin D-mediated processes at the tissue level. Moreover, because 1α-hydroxylase function is less tightly regulated in children than adults[157], a low serum 25(OH)D concentration in children may not be a major rate-limiting factor in vitamin D-mediated processes, until perhaps the concentration of the circulating substrate drops below a critical threshold. Unfortunately, we were not able to measure serum 1,25(OH)2D concentrations in the study participants, nor was it possible to collect pulmonary secretions to examine cytokine secretion or vitamin D metabolites in the respiratory milieu. In future research, the measurement of systemic and local alveolar concentrations of 1,25(OH)2D or 1α-hydroxylase function in the context of a viral ALRI may help to clarify this issue.

In contrast to the FokI SNP, we did not find that the TaqI polymorphism was associated with ALRI susceptibility. The accumulated body of literature examining the role of the TaqI SNP in bone mineralization suggests that there is no significant association[250]. Studies in adults have shown a protective effect of the t allele in pulmonary tuberculosis[169,211]. However, in the present data, there was no trend that would suggest that the study was underpowered to show a similar significant effect of the t allele in childhood ALRI. The reason for these discrepant findings is unclear, but may include a yet uncharacterized distinctness between the host cellular processes that respond to mycobacteria compared to viruses, or linkage of the SNP to unidentified genes implicated in immune defence. The TaqI SNP is in a coding region of the VDR gene, but does not alter the structure of the VDR and thus FokI SNPs may theoretically be more likely to lead to differences in clinical outcomes.

Several issues must be considered in the interpretation of the FokI and TaqI SNP data. First, we found that the FokI f and TaqI t alleles were in linkage disequilibrium, unlike most previous studies in which both polymorphisms were described. This does not affect the single-locus analysis conducted here, but would be important to consider if multi-loci haplotypes were analysed, not conducted in the present study because of the small sample size.

Second, the TaqI genotype distribution in participants with two parents of European descent (TT 45%, Tt 49%, tt 6%) was somewhat different from that previously published for Caucasian Canadian women (TT 37%, Tt 47%, and tt 16%)[166]. Although genotyping errors are possible, the results of the PCR-restriction fragment length polymorphism assay were unambiguous because of the large amount of extracted DNA used in the assay, and therefore we feel that the findings were unlikely to be due to laboratory error. More likely is that given the rarity of the tt genotype, the differences were an artefact of a relatively low sample size in the present study. In future studies of these SNPs, larger samples would be advisable to enable more precise estimates of allele frequencies and to undertake haplotype analysis.

The third issue was that in comparison to the FokI FF genotype, we found a strong association between the ff genotype and the risk ALRI (unadjusted OR 7.4) but could not discern an obvious intermediate effect of the heterozygous genotype (OR 1.3). One explanation for this finding would be that the f allele is recessive, not codominant, and thus both alleles would have to be present in order to cause a phenotypic difference. However, this explanation is not supported by previous laboratory[226] and clinical data[242,246] in which clear 'dose response' effects of the f allele have been observed. Therefore, the more likely reason was that the sample size was not large enough to demonstrate the statistical significance of a small increase in the risk of ALRI associated with the expression of only one f allele, particularly in the context of a generally vitamin D-replete population. Assuming the latter explanation to be true, we feel that it was justified to combine the FF and Ff groups for the purposes of analysis because of what appeared to be a very small effect of a single f allele. However, in future studies, larger samples sizes might help to discriminate the differential risks associated with one or two f alleles.

Social and environmental risk factors for ALRI

There were several social and environmental risk factors for ALRI in this population that were significant after controlling for participant age, including higher birth order among siblings, maternal smoking during pregnancy, exposure to a 'sick contact' (someone with symptoms of cough or runny nose) in the previous week, exposure to passive cigarette smoke (i.e., a higher number of smokers in the household where the child resides), a sibling history of asthma, lower socioeconomic status (i.e., paternal non-completion of high school), and greater household crowding. Higher birth order likely reflected the presence of older siblings in the home who readily transmit infection to younger children; in the present study, this risk was likely better accounted for by the parental report of the presence of a 'sick contact' in the home, which was retained in the final regression model. Although the present study was, to our knowledge, the first case-control study of early childhood ALRI risk factors in Canada, these risk factors were generally similar to those that have been previously reported for bronchiolitis in other developed countries[45].

A dramatic but predictable finding of this study was the greatly increased risk of ALRI in children of Aboriginal descent. Aboriginality did not emerge as a risk factor for ALRI simply because of a relatively low proportion of Aboriginal children undergoing elective surgery. In fact, the 42% of children with ALRI who had at least one parent of Aboriginal heritage represented an enormously disproportionate burden of illness relative to the proportion of the Alberta population under 15 years of age who are Aboriginal (8.7 %)[251], which closely reflected the proportion of control group participants with at least one Aboriginal parent (9 %). Furthermore, this finding was consistent with federal government data demonstrating that children aged 1 – 4 years from First Nations have an increased risk of hospitalization for respiratory illness compared to the general population[252].

There are many possible underlying reasons to explain why Aboriginal ethnicity is a risk factor for early childhood ALRI in Canada, since ethnic labels are merely markers for a complex array of socioeconomic, cultural, and biological determinants of health[253]. Although most Aboriginal children admitted to the general paediatric service at the SCH are of First Nations or Métis background[254], this study did not differentiate among the diversity of cultural groups encompassed by the term Aboriginal; therefore, conclusions cannot be uncritically applied to individual indigenous communities. Furthermore, there were very few participants from northern Canada in both study years, and therefore the results, including VDR genotype distributions, cannot necessarily be extrapolated to the Inuit population within which the highest rates of ALRIs have been observed[51].

Despite these important reservations, we observed a clustering of several ALRI risk factors that disproportionately affected those children for whom at least one parent/caregiver self-identified as Aboriginal, including maternal smoking during pregnancy, a higher number of smokers in the household, higher household crowding index, higher birth order, shorter duration of exclusive breastfeeding, lower participant age, and lower socioeconomic status (indicated by a lack of post-secondary education among the majority of Aboriginal parents/caregivers). Although the multivariate model that controlled for these social factors explained a substantial proportion of the risk associated with paternal Aboriginality (crude OR 8.9 versus adjusted OR 5.7), the crude odds ratio may be a more accurate measure of

this profound social inequity[255]. Furthermore, the strength of the association after adjustment suggests that many factors were unaccounted for by the measured social factors.

Given that the increased ALRI risk associated with the VDR ff genotype was independent of ethnicity, it is appealing to cautiously speculate that the greatly increased risk of ALRI among Aboriginal children is at least in part due to a genetic factor. The prevalence of the ff genotype among participants for whom both parents were Aboriginal (25 %) was more than double the prevalence in children for whom both parents were of European descent (11 %). The reliability of this data is suggested by the parallels between the genotype distributions among the Caucasian children in the present study (FF 35%, Ff 54%, ff 11%) and those in a US study (FF 39%, Ff 45%, ff 16%)[242], and between the Aboriginal children in our study (FF 15%, Ff 60%, ff 25%) and Mexican-American children (19%, 56%, 25%)[242], the only other native-North American population for which data are available. As further comparison, the ff genotype is very rare in African-Americans (FF 65%, Ff 31%, ff 4%)[256], whereas the allelic distribution among mixed-lineage Peruvians is essentially inversed (FF 7%, Ff 36%, ff 57%)[169]. It is beyond the scope of this discussion to elaborate on the evolutionary factors that could account for these intriguing differences, but it is interesting to consider why those who migrated from Eurasia to the Americas approximately 15 000 years ago experienced selective pressures in favour of what appears to be a less efficient VDR variant. Future research correlating functional vitamin D status and health outcomes within diverse American indigenous populations could help to further illuminate these observations.

Predictors and patterns of vitamin D status in Canadian children under two years of age

This study was not primarily designed to examine the vitamin D status of young children and infants in the general Canadian population. However, because the study provides the only existing Canadian data on vitamin D status in post-neonatal infancy and early childhood (aside from reports of children with rickets), the observed trends are worthy of mention. Overall, the participants in both the case and control samples were universally more vitamin D replete than any other age group in Canada previously studied (i.e., neonates[100], older children and adolescents[120], adults[116]). In contrast to the high prevalence of VDI-C in older children and adolescents in our earlier study[120], almost all of the children in the present study appeared to be consuming enough vitamin D to avoid VDI-C. There were only 4 participants with 25(OH)D < 40 nmol/L, of whom 3 were exclusively-breastfed infants not receiving vitamin D supplementation, a finding consistent with previous observations of unsupplemented breastfed infants born in northern countries who rapidly deplete fetal stores of vitamin D in the neonatal period[133]. All of the other exclusively-breastfed infants (7/10) were receiving a vitamin D supplement; regardless of the frequency or dose, these supplemented infants all maintained 25(OH)D concentrations > 40 nmol/L.

Despite the nearly universal absence of VDI-C in this population, there were many children who had VDI-I, even among those who met the recommended 'adequate intake' (5 mcg/day). This suggests that if future research were to establish that the normal early childhood 25(OH)D range is > 80 nmol/L, dietary recommendations will likely need to be

altered to ensure that most of the population achieves vitamin D sufficiency, similar to that which has been proposed for adults[89].

In an analysis that included cases and controls, the strength of the association between vitamin D status and weight-adjusted vitamin D intake (Spearman $\rho = 0.319$) was similar to that which we observed in older children and adolescents[120]. The weakness of the relationship is at least in part attributable to parent/caregiver imprecision in the recall of their child's intake, the constantly changing nature of a child's diet in the first years of life, the lack of measurement of the vitamin D intake from breast-milk, and the lack of accounting for factors that mediate cutaneous vitamin D production (e.g., duration of time exposed to sunlight during the summer, sunscreen use, swaddling or clothing practices, etc). Two of the other predictors of 25(OH)D concentration could be readily explained by confounding variables: maternal Aboriginal heritage was associated with younger participants who generally had higher 25(OH)D levels because of fortified infant formula intake; those not classified as 'exclusively breast-fed' were formula-fed infants who generally received high doses of vitamin D. Infant formula and vitamin D supplementation were significantly associated with increased 25(OH)D level, whereas milk intake was associated with lower levels because milk drinkers were almost universally children over the age of one year who had relatively low amounts of vitamin D in their diets compared to the younger children who fed infant formula or receiving a vitamin D supplement. Predictably, 25(OH)D concentration slightly decreased with greater time passed since the summer, as vitamin D stores are depleted through the winter months during which cutaneous vitamin D is not produced[117].

Unlike older children and adolescents, among whom vitamin D sources are often scarce, the vast majority of the participants in this study were formula-fed infants or children who regularly consumed vitamin D-fortified milk (cow's milk or soy-based). Therefore, from a broad perspective, the high 25(OH)D levels observed in this young population can be attributed to their relatively high average weight-adjusted estimated dietary vitamin D intake (1.2 mcg/kg/day) which was much greater than in the older children and adolescents in our previous study, using the same food frequency questionnaire (0.43 mcg/kg/day in children aged 2 – 8 years; 0.22 mcg/kg/day in children aged 9 to 16 years)[120].

Our cross-sectional data provided a picture of the progression of vitamin D status during the first two years of life that was very consistent with previous cross-sectional[240] and longitudinal studies[257,258] of infants receiving dietary vitamin D. Although many newborns initially have low 25(OH)D concentrations due to poor maternal stores[147], we observed that serum 25(OH)D concentrations were often high during the first several post-neonatal months, likely as a result of a high per-kilogram dose of exogenous vitamin D in fortified infant formula or a commercial supplement. However, vitamin D intake substantially decreased from a mean of approximately 1.5 mcg/kg/day in the first year of life to about 0.8 mcg/kg/day in the second year. This drop can likely be explained by the transition to fluid milk, which contains a lower concentration of vitamin D3 than infant formula and is consumed in lower volumes on a per-kilogram body weight basis compared to infant formula. The effect of the observed pattern of vitamin D status through the first two years of life was not reflected in a simple linear decrease in 25(OH)D concentration as age increased. Rather, the pattern was more realistically, though imperfectly, reflected in a

bimodal distribution whereby 25(OH) concentrations subtly declined with increasing age during the second year (Figure 2). This trend also suggests that in this age group, summer sun exposure was a very minor factor in determining wintertime vitamin D status, since children in their second year of life would have experienced at least one summer, whereas many of the infants would never have been exposed to summer sun, particularly those under 6 months of age at the time of participation. Based on our previous observations, the decline in vitamin D status that starts in the second year of life appears to continue throughout childhood as vitamin D intake (per kilogram body weight) diminishes, eventually leading to VDI in many adolescents[120].

Methodological limitations

This study had several methodological limitations that affected the interpretation of the findings. First, as described in Chapters Two and Three, we encountered significant unexpected barriers to the use of a single uniform 25(OH)D assay. Ultimately, the primary analysis involved radioimmunoassay (RIA) results from year one, and LC/MS/MS results from year two. In order to standardize the results, we applied a conversion factor calculated during a period in which both assays were routinely used in the UAH laboratory (C. Prosser, personal communication). In general, both methods are highly correlated, but the RIA tends towards a negative bias, particularly at higher 25(OH)D levels. Despite the use of the conversion factor, the standardized year-one results tended to be lower than the raw second-year LC/MS/MS data (72.9 versus 82.9 nmol/L). In such a young population, this was unlikely to be accounted for by differences in summertime sun exposure, as discussed above, and was more likely due to differences in the assays not fully accounted for by the conversion factor.

The reliability and inter-assay comparability of laboratory measurements of 25(OH)D has been a long-standing concern in vitamin D research[259]. Comparisons among institutions using different protocols and assays have led to wide inter-labatoratory variability[260]. However, since 1990, efforts to create international standards have minimized the inconsistencies. The UAH laboratory currently participates in an international network called the Vitamin D External Quality Assessment Scheme (DEQAS)[261], which allows laboratories to compare their results to those of other labs throughout the world. Using both the RIA and LC/MS/MS, the UAH laboratory performs well in relation to international standards (C. Prosser, personal communication). Nonetheless, we were unable to directly compare the RIA results from years one and two because the second-year RIA kit had a significant negative bias. Although control samples confirmed intra-assay precision, the year-to-year discrepancy was most likely accounted for by differences in reagent and material lots (C. Prosser, personal communication).

To further complicate the comparison of RIA and LC/MS/MS results, one lab using a LC/MS/MS method similar to that used in this study recently reported high concentrations of C-3 epimers of 25(OH)D in samples from about 20% of infants under the age of one, but not in older patients[262]. These epimers were not detected by the Diasorin RIA, and thus led to significant discrepancies between the results from RIA and LC/MS/MS for infant samples. Although the UAH method is not set up to distinguish C-3 epimers from non-

epimerized 25(OH)D, it is possible that circulating epimers in samples from many of the infant participants led to high levels measured by the LC/MS/MS that would not be accounted for by the RIA–to–LC/MS/MS conversion factor based on data from primarily adult serum samples. Since naturally-occurring C-3 epimers have been shown to be biologically active[263], a serum measurement that includes the epimers may be a more accurate reflection of vitamin D status in infants.

Despite the methodological limitations, a sensitivity analysis using combinations of raw and standardized data demonstrated that regardless of the standardization approach, there was no significant difference in vitamin D status between case and controls, particularly after adjusting for the much younger age of cases. In the unadjusted analysis, the significantly lower mean 25(OH)D concentration in cases in the second year of the study was strongly skewed because there were so few cases recruited in the second year, of whom a high proportion (3/8) were unsupplemented breast-fed infants.

An additional issue with respect to the assessment of vitamin D status related to the potential modifying effect of systemic inflammation on serum 25(OH)D levels. Overall, the absence of a difference in mean 25(OH)D concentration between cases and controls, and the lack of an apparent effect of duration of illness on 25(OH)D concentration among ALRI cases, suggested that serum 25(OH)D concentrations were unaltered by the inflammatory response, consistent with previous findings[86]. Although unlikely, it remains possible that the results reflected two phenomena that cancelled one another – a predisposition to infection due to VDI, but a rise in the 25(OH)D concentration due to the acute phase response, thus concealing a pre-existing deficiency state. Only a prospective cohort study in which vitamin D status is assessed before the onset of infection would resolve this issue. We also considered a modified case-control design in which vitamin D status would be measured after ALRI resolution, but logistical issues (e.g., many children do not live in the city of Edmonton) prevented a study of this nature.

Further limitations were related to the case-control study design. The selection of an appropriate control group was challenging, as discussed in Chapter Two. As well, the impracticality of age-matching the participants led to heterogeneity between the case and control groups that may have been unrelated to ALRI susceptibility. Statistical techniques permitted conventional adjustment for a variety of confounding factors, yet this analysis was obviously limited to those variables that were assessed in a parental/caregiver questionnaire. Reassuringly, the ALRI risk factors identified in this study were clinically reasonable and consistent with previous studies, reinforcing the validity of the vitamin D-related observations. The concentrated clustering of risk factors we observed in this study led to difficulties in deciphering which factors were most directly related to disease risk. However, even in the absence of evidence of direct relationships, a case-control study allows the characterization of high-risk populations within which public health actions can be focused. In addition, identification of risk factors points investigators towards potential causal factors, allowing focused design of trials of preventive or therapeutic interventions[264].

Case-control studies have long been a standard approach to the identification of genetic associations with disease outcomes, yet it has been recognized that results from an initial report are often not reproduced in studies of other populations, suggesting a high rate of false-positive claims[265]. Although it is important to interpret our finding of the FokI SNP

association with ALRI risk in light of the limitations of the case-control study design, the finding is substantiated by its biological plausibility based on current knowledge of the effect of the f allele on phenotype, the persistence of association after accounting for the possible effects of population stratification (i.e., ethnic bias), and the finding that the association remained significant in an analysis that adjusted for random confounding variables. However, it is important to acknowledge the wide confidence interval around the odds ratio, which may have been spuriously high due to a relatively small study sample; a larger study would be necessary to provide a more accurate risk estimate.

Conclusions

In summary, serum 25(OH)D concentration was not associated with the risk of ALRI in young children and infants, but there was a significant association of ALRI susceptibility with a SNP in the translation start-site of the gene encoding the VDR. The public health implication that emerges from these findings is that given the relatively replete vitamin D status in this sample of Canadian children aged 1 month to 2 years, efforts to further increase the amount of vitamin D supplementation are unlikely to alter the risk of early childhood ALRI in the general population. However, the association of the VDR FokI ff genotype with ALRI susceptibility provided intriguing evidence that vitamin D may indeed be implicated in the host immune response, as would be expected based on accumulating laboratory research. Further studies are necessary to confirm the association between vitamin D and the host response in ALRI and clarify its cellular and biochemical mechanisms, ideally by integrating measures of vitamin D stores, rates of conversion of circulating forms of vitamin D to the active metabolite, and VDR responsiveness, to enable the comprehensive measurement of functional vitamin D status. This study was not able to determine whether specific target populations, including unsupplemented breast-fed infants or children with a combination of suboptimal vitamin D status and the FokI ff genotype, would benefit from vitamin D supplementation from the standpoint of ALRI prevention, at least at the daily dose currently recommended (400 IU in the first year of life, 200 IU per day thereafter). The highest yield of data would likely be produced in research efforts that focus on children in Canadian communities that are burdened by the highest risks of childhood ALRI, such as the Inuit in northern Canada.

Despite the flurry of adult studies addressing the newly-recognized physiologic roles of vitamin D, the pediatric population remains relatively unstudied from the standpoint of extra-skeletal effects of VDI. Given that the young age group studied here demonstrated relatively adequate vitamin D status compared to older children and adolescents, future research should focus on potential associations between vitamin D status and infectious or inflammatory disease states in the pediatric populations that are at the highest risk of suboptimal vitamin D status.

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Appendix A - Case Eligibility Form

	Criteria	YES	NO	UNKNOWN
INCLUSION CRITERIA (eligibility requires a 'Yes' response to each item)	**Age:** > 1 month (30 days) and < 2 years (up to day prior to second birthday) at the time of admission (use corrected age for premature infants).			
	Diagnosis: Admitted to the Stollery Children's Hospital inpatient ward with an acute lower respiratory tract infection (ALRI) that fulfils criteria for at least one of the three ALRI case definitions.			
EXCLUSION CRITERIA (eligibility requires a 'No' response for each item)	Acute pulmonary aspiration event prior to admission (based on history and chest x-ray findings consistent with pulmonary aspiration)			
	Foreign body in respiratory tract during this admission			
	Pulmonary aspiration syndrome: chronic or recurrent aspiration treated by enteral tube feeding to bypass swallowing mechanism (nasogastric, nasojejunal, or gastrostomy tube)			
	Supplemental oxygen use at home for any reason			
	Symptomatic congenital heart disease (unrepaired cyanotic heart disease or congestive heart failure)			
	Tracheostomy			
	Congenital lung abnormality (e.g., hypoplastic or dysplastic lung)			
	Chronic interstitial lung disease (e.g., pulmonary hemosiderosis, lymphocytic interstitial pneumonitis)			
	Cystic fibrosis			
	Neurological disorder that compromises the strength of the respiratory muscles (e.g., spinal muscular atrophy)			
	Anatomical abnormality that leads to pulmonary aspiration of oral or gastric secretions (e.g., tracheoesophageal fistula, esophageal web, laryngeal cleft, vocal cord paralysis).			
	Congenital or acquired immunodeficiency			
	Immunosuppressive chemotherapy			
	Hematologic malignancy (e.g., leukemia)			
	Sickle cell disease			
	Tuberculosis			

Appendix B - Control Participant Eligibility Form

	Criteria	YES	NO	UNKNOWN
INCLUSION CRITERIA (eligibility requires a	**Age-matched** with case participant: +/- 60 days of the age of the matched case			
	Sex-matched with case participant			
	Elective admission to the hospital short-stay observation unit (5F5), the day surgery unit, or the radiology department for elective surgery, procedure or investigation			
	Recruited within 2 weeks of the matched case.			
EXCLUSION CRITERIA (eligibility requires a 'NO' response to each item)	Any **overnight** admission to hospital for 'asthma', 'reactive airway disease', 'bronchiolitis', 'RSV', 'bronchitis', 'pneumonia', 'bronchopneumonia', or 'chest infection'.			
	Currently fulfills the criteria for any of the three case definitions for ALRI.			
	Pulmonary aspiration syndrome: chronic or recurrent aspiration treated by enteral tube feeding to bypass swallowing mechanism (nasogastric, nasojejunal, or gastrostomy tube)			
	Supplemental oxygen use at home for any reason			
	Symptomatic congenital heart disease (unrepaired cyanotic heart disease or congestive heart failure)			
	Tracheostomy			
	Congenital lung abnormality (e.g., hypoplastic or dysplastic lung)			
	Chronic interstitial lung disease (e.g., pulmonary hemosiderosis, lymphocytic interstitial pneumonitis)			
	Cystic fibrosis			
	Neurological disorder that compromises the strength of the respiratory muscles (e.g., spinal muscular atrophy)			
	Anatomical abnormality that leads to pulmonary aspiration of oral or gastric secretions (e.g., tracheoesophageal fistula, esophageal web, laryngeal cleft, vocal cord paralysis).			
	Congenital or acquired immunodeficiency			
	Immunosuppressive chemotherapy			
	Hematologic malignancy (e.g., leukemia)			
	Sickle cell disease			
	Tuberculosis			

Appendix C

Information Sheet

Title of Study: **Vitamin D Status among Infants and Children with Acute Lower Respiratory Tract Infections**

Investigators: Dr. Daniel Roth, Department of Pediatrics (ph. 407-3339)
Dr. Adrian Jones, Department of Pediatrics
Dr. Joan Robinson, Department of Pediatrics
Dr. Sunita Vohra, Department of Pediatrics
Dr. Connie Prosser, University of Alberta Hospital Laboratory
Mr. Ben Vandermeer, Alberta Research Centre for Child Health Evidence

Purpose of the study:

We are doing this study to find out if children with lung infections (bronchiolitis or pneumonia) have lower vitamin D levels compared to children who have not had lung infections. If this is found to be true, then in the future, we might be able to lower the chance that children get these diseases by giving them extra vitamin D.

Background information:

Vitamin D is sometimes called the "sunshine vitamin." It is made by skin that is exposed to sunlight during the summer. It is also found in a few foods. We know that many children in Alberta do not make enough vitamin D during the winter. That is why doctors suggest that breast-fed babies receive extra vitamin D by mouth. Commercial infant formulas also contain extra vitamin D.

Vitamin D is needed for proper bone growth. Without enough vitamin D, children can develop the serious bone disease called 'rickets'. Scientists have also begun to learn more about the role of vitamin D in other systems in the body. We now know that vitamin D affects the way that the body resists infections.

For this study, we need to involve children with and without lung infections. This Information Sheet contains specific information about the study, and what your child's participation would involve.

Study Procedures:

If your child takes part in this study, we will ask you some questions about your child's current and past health. Other questions ask for details about the family, including the mother and father's education levels. We will also ask you about the foods your child eats

in an average week. We will also measure your child's weight and height. If your child is now in hospital, we will collect information about the length of your child's hospital stay, and the amount of time he/she needed extra oxygen.

We will collect a small sample of your child's blood. This blood will be used to do a lab test that measures the vitamin D level. The total amount of blood is 2 mL, which is about one-half teaspoon. If your child needs another blood test, is having an intravenous line placed, or will be asleep under anaesthesia for a procedure, then we can collect the blood sample at that time. If not, then blood collection will involve insertion of a tiny needle into a vein just under the skin. After only a few seconds, the tubes of blood are filled. The needle is then removed from the skin right away. The time required for all of these procedures and questions is about one hour.

Benefits:

The main benefit of this study is that we will learn about the link between vitamin D and lung infections. The benefit to your child is that we may detect a low level of vitamin D that you did not know about before. This can be treated with a change in your child's diet or vitamin D supplements, if necessary.

Risks/Inconveniences:

If a needle is used to collect the blood, then this may cause brief and mild pain. It also has a low risk of causing a small bruise at the collection site. For children older than 6 months, we may offer to use a local skin freezing cream (e.g., EMLA) if you wish, to reduce the pain. The possible inconvenience to you is the time (about one hour) required for completing the questionnaire, the diet history, and recording your child's height and weight.

Confidentiality:

Only the doctors conducting this study will have access to the facts collected about your family or child. Surveys will be stored in a secure cabinet in the Department of Pediatrics at the University of Alberta. Although the results of this study may be presented publicly, your family or child will never be identified in any way. By signing the consent form you give permission to the study staff to access any personally identifiable health information which is under the custody of other health care professionals as deemed necessary for the conduct of the research.

With your permission, we will send the result of the vitamin D blood level to your family doctor or pediatrician. After the study is over, we may keep your child's blood sample in the lab in case we need to test it for other minerals, such as calcium, iron, or zinc. These tests would be done without anyone knowing the name of the person who gave the blood sample. This means that you would not be contacted with any results.

Freedom to refuse or withdraw from the study:

It is your choice whether or not your child takes part in this study. You are free to refuse to let your child take part in this study. You are free to withdraw your child from the study at any time without any effect on your child's health or the care you or your child will receive now or in the future.

Additional contacts:

If you have any concerns about any aspect of this study, you may contact the Patient Concerns Office of Capital Health at 407-1040. This office has no connection with the study investigators.

Consent Form

Vitamin D Status among Infants and Children with Acute Lower Respiratory Tract Infections

For each question, please check the box next to the response that applies to you. These questions apply to the information you received from the study investigator or designee and what you have read on the first two pages of this consent form.

1. Do you understand that your child has been asked to be in a research study about vitamin D and respiratory infections in infants and children?
   ☐Yes ☐No

2. Have you read the attached information about the study?
   ☐Yes ☐No

3. Do you understand the benefits and risks to your child by his/her taking part in this research study?
   ☐Yes ☐No

4. Have you and/or your child had a chance to ask questions and discuss this study?
   ☐Yes ☐No

5. Do you understand that you are free to refuse to participate in the study, and can withdraw your child from the study at any time?
   ☐Yes ☐No

You do not have to give a reason. Refusal or withdrawal will not affect your child's health care.

6. Has "confidentiality" been explained to you? Do you understand who will have access to your child's study records?
   ☐Yes ☐No

7. Do you want the investigators to send results of the vitamin D test to your family doctor or your child's pediatrician after the end of the study? **It may take about one month, or longer, for the results to become available.*

   ☐Yes ☐No

If so, please provide the name and address, fax number, or phone number of your child's main community doctor:

_____

_____

This study was explained to me by: _____

Name of child taking part in study: _____

By signing below, I agree to allow my child to take part in this study.

_____	_____	_____
Signature of Parent/guardian	Date	Witness
_____		_____
Printed Name		Printed Name

I believe that the parent/guardian signing this form understands what is involved in the study and voluntarily agrees to the child's participation.

_____	_____
Signature of Investigator or Designee	Date

Information and Consent Sheet

Title of Study: **Vitamin D Status among Infants and Children with Acute Lower Respiratory Tract Infections**

Investigators: Dr. Daniel Roth, Department of Pediatrics (pg. 407-3339)
Dr. Adrian Jones, Department of Pediatrics
Dr. Joan Robinson, Department of Pediatrics
Dr. Sunita Vohra, Department of Pediatrics
Dr. Connie Prosser, University of Alberta Hospital Laboratory
Mr. Ben Vandermeer, Alberta Research Centre for Child Health Evidence

OPTIONAL GENETIC TESTING

Thank you for agreeing to your child's participation in the vitamin D and lung infection study. As part of this study, we are using some of the collected blood to do a genetic test related to vitamin D. A gene is a piece of information inherited from the child's parents. We will look at one gene that controls how the body uses vitamin D. We want to find out if this gene affects how well a person fights off lung infections. Based on what scientists know now, the result of this test does not have any meaning for your child or family's current or future health. So, your child's personal test result will not be useful to you or any of your child's doctors. But, we will look at the results from many children together to answer our study question.

This blood sample will be collected at the same time as the collection of the other blood sample, so will NOT involve any extra needle pokes. The amount of blood we will collect is 2 mL, which is about one-half teaspoon.

Benefits:

The main benefit of this study is that we will learn about the link between vitamin D genes and lung infections in children. There is no direct benefit to your child.

Risks/Inconveniences:

There are NO risks to your child by taking part in the genetic testing. There are no inconveniences because the sample is collected at the same time as the other vitamin D sample.

Confidentiality:

Only the doctors conducting this study will have access to the results of this test. Although the study findings may be presented publicly, your family or child will never be identified in any way. We will NOT send the results of the genetic test to your child's physician, because the result does not affect your child's health in any way that we currently know. By signing the consent form you give permission to the study staff to access any personally identifiable health information which is under the custody of other health care professionals as deemed necessary for the conduct of the research.

Freedom to refuse or withdraw from the study:

It is your choice whether or not your child takes part in this study. You are free to refuse to let your child take part in the genetic study even if he/she participates in the other parts of the vitamin D study. You are free to withdraw your child from the study at any time without any effect on your child's health or the care you or your child will receive now or in the future.

Additional contacts:
If you have any concerns about any aspect of this study, you may contact the Patient Concerns Office of Capital Health at 407-1040. This office has no connection with the study investigators.

Do you agree to the genetic test described in this information sheet?

☐ Yes ☐ No

The genetic testing was explained to me by: _____
Name of child taking part in study: _____

By signing below, I agree to allow my child to take part in the vitamin D gene testing.

_____ _____ _____
Signature of Parent/guardian Date Witness

_____ _____
Printed Name Printed Name

I believe that the parent/guardian signing this form understands what is involved in the study and voluntarily agrees to the child's participation.

_____ _____
Signature of Investigator or Designee Date

Appendix D

Vitamin D - ALRI Study Questionnaire

1. Child's birthdate (day/month/year)	____ (DAY)/_____(MONTH)/_____(YEAR)
2. Sex of child	a) Male | b) Female
3. What is the major ancestry or ethnicity (i.e., family background) of your child's biological MOTHER?	a) Aboriginal Canadian (First Nations or Inuit or Métis) b) Caucasian from Europe or the former Soviet Union c) Chinese or East Asian d) Indian (Asian subcontinent) e) Hispanic or Central/South American f) African g) Middle Eastern h) Other i) If other, please specify: _____
4. What is the major ancestry or ethnicity (i.e., family background) of your child's biological FATHER?	a) Aboriginal Canadian (First Nations or Inuit or Métis) b) Caucasian from Europe or the former Soviet Union c) Chinese or East Asian d) Indian (Asian subcontinent) e) Hispanic or Central/South American f) African g) Middle Eastern h) Other i) If other, please specify: _____
5. Location of your child's current main residence:	City/town:_____ To be classified by interviewer according to latitude as: a) South of Edmonton b) In Edmonton (52°) or north to, but not including, Ft. McMurray (56°). c) Ft. McMurray (56°) and north to, but not including, Yellowknife (62°) d) Yellowknife (62°) and north e) Unknown
6. *For children for whom aboriginal heritage is identified:* Does your child live on a reservation?	a) Yes, lives ON reservation most of the time b) No, lives OFF reservation most of the time c) Child is not aboriginal d) Unknown

7. How many days has your child spent in a southern* 'sunny' location since October? (*south of Los Angeles)	_____ days		
8. How many times has your child been admitted overnight to hospital due to "pneumonia", "bronchiolitis", "RSV", "bronchitis", "bronchopneumonia", or a "chest infection" (INCLUDING the current episode for cases)? All controls should have the response '0'	_____ (# of hospitalizations)		
9. Has your child ever been diagnosed with 'asthma' or "reactive airways disease" by a physician or nurse?	a) Yes	b) No	c) Unknown
10. Has your child ever had 'eczema' (chronic dry itchy skin) diagnosed by a physician or nurse?	a) Yes	b) No	c) Unknown
11. How many separate episodes of "ear infections" has your child had diagnosed by a physician or nurse since birth? *If both ears were affected at the same time, this is considered to be one single episode.	_____ (# episodes)		
12. Gestational age at birth (the number of weeks the baby was in the womb)	____ weeks		
13. Birthweight	___ lbs and ____ oz OR _____ kg		

14. *For children at least 2 months old:* When did your child last receive an immunization or vaccine shot? Age_____ Status to be assessed by nurse.	a) Immunizations up-to-date b) Immunizations delayed (≥ 1 month older than age of the most recent scheduled immunization appointment that the child did not attend) c) No immunizations since birth (excluding those given to newborn, e.g., BCG) d) Unknown		
15. How was your child being fed at home prior to admission?	a) Breast-feeding only	b) Oral feeding (other than exclusive breast-feeding)	c) Enteral tube (nasogastric, nasojejunal, gastrostomy) - supplement or exclusively
16. For how many weeks since birth was/has the child exclusively breast-fed? "Exclusive" = breast-milk is the only food or drink provided to the infant	_____ (# of weeks)		
17. Does your child have any of the following conditions?	a) No, none of the following b) Chronic kidney problems c) Chronic liver problems d) Celiac disease or inflammatory bowel disease e) Use of anti-convulsant (i.e., anti-seizure) medications f) Use of rifampin or isoniazid		
18. Birth order among siblings	_____		
19. How many cigarette smokers live in the same house as your child?	_____ (# of smokers)		

20. Did the child's mother smoke any cigarettes during the pregnancy with this child?	a) Yes	b) No	c) Unknown
21. What type of heating appliance or furnace is used inside the house where your child lives?	a) Electric radiator b) Hot water c) Gas furnace (e.g., forced-air) d) Wood-burning stove e) Other		
22. Does the child currently attend daycare regularly? (at least 1 full day per week)	a) Yes	b) No	c) Unknown
23. Mother (biological) has a history of physician-diagnosed asthma?	a) Yes	b) No	c) Unknown
24. Father (biological) has a history of physician-diagnosed asthma?	a) Yes	b) No	c) Unknown
25. Any full- or half-sibling (biological) with a history of physician-diagnosed asthma?	a) Yes	b) No	c) Unknown
26. Was the child in contact with, or cared for, by anyone with a NEW "cold" or "cough" or "flu" in the 1 week prior to admission?	a) Yes b) No c) Unknown		
27. What is the highest level of education obtained by your child's mother (or primary legal caregiver)?	a) College or university degree COMPLETE b) College or university degree INCOMPLETE c) High school COMPLETED (high school diploma) d) High school INCOMPLETE e) Unknown		
28. What is the highest level of education obtained by your child's father?	a) College or university degree COMPLETE b) College or university degree INCOMPLETE c) High school COMPLETED (high school diploma) d) High school INCOMPLETE e) Unknown		
Total number of people living in the house where the child lives	29. Adults (18 years old and older): _____ 30. Children (17 years old and younger): _____		
31. Total number of bedrooms in the house where the child lives (bedroom = any room in which people regularly sleep)	____ rooms		

Appendix E

Food Frequency Questionnaire

Describe the amount of each listed food item consumed during a typical week (7 days) prior to the onset of the acute illness that has resulted in the current hospitalization (for cases), or currently (for controls).

Item	Quantity – Frequency – Brand	Vitamin D (mcg)
Infant formula (1 oz = 28 mL)		
Vitamin D supplement		
Fluid milk (1 cup = 250 ml = 8 oz)		
Canned milk (1 cup = 250ml = 8 oz)		
Skim milk powder (25 g = 2 tbsp.)		
Yogurt (1/2 cup = 100 g)		
Margarine (1 teaspoon = 5 ml)		
Salmon (50 g = ½ can = 1 ¾ oz)		
Tuna (50 g = ½ can = 1 ¾ oz)		
Other Fish (50g = 1 ¾ oz)		
Vector Cereal (1 cup = 8 oz)		
Vector Cereal Bar (1 bar)		
Liver (100 g = 3 ½ oz)		
Egg yolks (1 yolk)		
Other supplements		
	TOTAL	

Appendix F

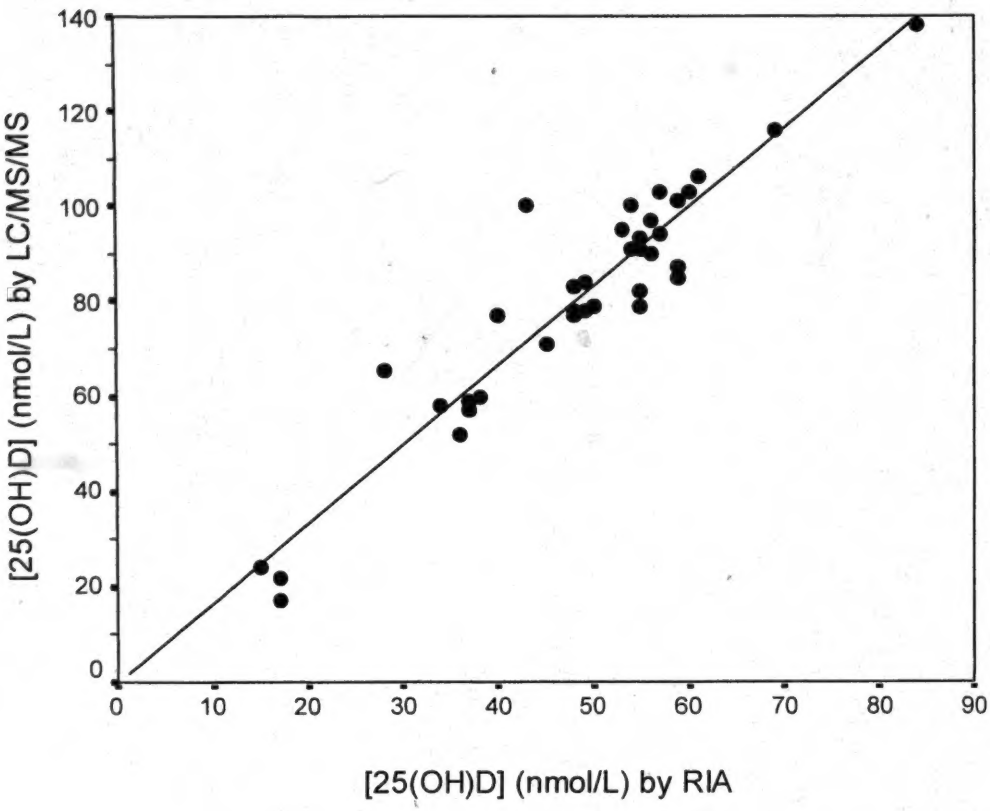
Additional data not included in the main body of text

a. Case-Control Comparison of Vitamin D Status and VDR genotype distributions after exclusion of 3 control participants who were older than 24 months of age.

Vitamin D status or VDR genotype	Number of participants with available data (Cases, Controls)	All Participants	Cases	Controls	*P* value
25(OH)D concentration (nmol/L)	64, 62	77.5 (SD 22.4)	77.0 (SD 24.2)	77.9 (SD 20.6)	0.829
Prevalence of vitamin D insufficiency (VDI-I) [25(OH)D < 80 nmol/L]	64, 62	67 (53.2 %)	33 (51.6 %)	34 (54.8 %)	0.425
Prevalence of vitamin D insufficiency (VDI-C) [25(OH)D < 40 nmol/L]	64, 62	4 (3.2 %)	3 (4.7 %)	1 (1.6 %)	0.322
VDR TaqI genotype1 TT Tt tt	56, 61	 54 (46.2 %) 55 (47.0 %) 8 (6.8 %)	 24 (42.9 %) 28 (50.0 %) 4 (7.1 %)	 30 (49.2 %) 27 (44.3 %) 4 (6.6 %)	0.790
VDR FokI genotype1 FF Ff ff	56, 61	 37 (31.6 %) 64 (54.7 %) 16 (13.7 %)	 14 (25.0 %) 29 (51.8 %) 13 (23.2 %)	 23 (37.7 %) 35 (57.4 %) 3 (4.9 %)	**0.012**

b. **Linear relationship between 25-hydroxyvitamin D concentrations [25(OH)D] measured by radioimmunoassay (RIA) and Liquid Chromatography – Tandem Mass Spectrometry (LC/MS/MS) in samples from the second year of the study (N = 36), R = 0.944, $P < 0.001$. The linear regression equation was:**

$$[25(OH)D]_{LC/MS/MS} = 1.67*([25(OH)D]_{RIA\text{-}YR2}) - 0.006$$



c. Associations between VDR TaqI genotypes and 25(OH)D concentration, maternal ethnicity, and paternal ethnicity (analysis includes cases and controls).

Characteristic	VDR TaqI Genotype			
	TT	Tt	tt	*P* value
Mean 25(OH)D (nmol/L)	76.3 (SD 24.8)	78.2 (SD 21.2)	75.3 (SD 14.7)	0.884
Maternal Ethnicity				
Aboriginal	14 (56.0 %)	9 (36.0 %)	2 (8.0 %)	0.521
European	39 (44.3 %)	43 (48.9 %)	6 (6.8 %)	
Paternal Ethnicity				
Aboriginal	16 (59.3 %)	10 (37.0 %)	1 (3.7 %)	0.325
European	37 (43.0 %)	43 (50.0 %)	6 (7.0 %)	

d. Confounding associations between paternal/maternal ethnicity and ALRI risk factors (includes cases and controls)

Risk Factor	Maternal Ethnicity		*P* value	Paternal Ethnicity		*P* value
	Aboriginal	Non-Aboriginal		Aboriginal	Non-Aboriginal	
Maternal Aboriginal ethnicity	-	-	-	20 (74.1 %)	6 (5.9 %)	**< 0.001**
Paternal Aboriginal ethnicity	20 (76.9 %)	7 (6.8 %)	**< 0.001**	-	-	-
Maternal high school non-completion	15 (60.0 %)	15 (14.7 %)	**< 0.001**	16 (61.5 %)	13 (13.0 %)	**< 0.001**
Paternal high school non-completion	15 (57.7 %)	17 (16.8 %)	**< 0.001**	19 (70.4 %)	13 (13.0 %)	**< 0.001**
Maternal smoking during pregnancy	18 (69.2 %)	23 (22.5 %)	**< 0.001**	17 (63.0 %)	23 (23.0 %)	**< 0.001**
Median number of smokers in the household	2.0 (IQR 2.3)	0.0 (IQR 1.0)	**< 0.001**	2.0 (IQR 3.0)	0.0 (IQR 1.0)	**< 0.001**
Median household crowding index	1.8 (IQR 1.0)	1.3 (IQR 0.5)	**< 0.001**	1.8 (IQR 0.8)	1.3 (IQR 0.5)	**< 0.001**
Median birth order	3.0 (IQR 2.0)	2.0 (IQR 2.0)	**0.004**	3.0 (IQR 2.0)	2.0 (IQR 2.0)	**0.002**
Median duration of exclusive breastfeeding (index, where 1 = 6 months)	0.0 (IQR 0.27)	0.25 (IQR 0.94)	**0.011**	0.0 (IQR 0.42)	0.23 (IQR 0.81)	0.108
Mean age (months)	8.7 (SD 5.3)	11.4 (SD 7.1)	0.085	8.2 (SD 5.7)	11.5 (SD 7.0)	**0.023**

Appendix G

Clinical Data Collection Form – CASE

	Item	Value
Clinical Data on admission	1. Respiratory Rate (breaths/minute) When first assessed in the emergency department	_____ breaths per minute
	2. Temperature Highest temperature recorded during the first 24 hours in hospital (including emergency department and ward)	____ ° C
	3. Oxygen saturation (on room air) In the emergency department when first assessed	____ %
	4. Meets clinical case definition for:	a) Bronchiolitis b) Pneumonia c) Respiratory distress NOS
Anthropometric Data	5. Weight	_____ kg
	6. Length	_____ cm
Historical Data (from chart)	7. Illness duration prior to admission to hospital (presence of symptoms of respiratory tract disease or fever)	_____ days
	Date and time of discharge from hospital	8. Date (mm/dd/yr): ___ / __/__ 9. Time (24-hour): _____
	Date and time of first recording of 'room air' on nursing chart preceding at least 24 hours free of supplemental oxygen requirements, or lasting until discharge (whichever is first).	10. Date (mm/dd/yr): ___ / ____ /___ 11. Time (24-hour): _____
	12. Did patient receive at least one dose of oral or intravenous steroids for the treatment of this acute illness?	a) Yes b) No c) Unknown
	13. Did the patient receive any antibiotics for the treatment of this acute respiratory illness?	a) Yes b) No c) Unknown
Microbiological Data	14. Results of the nasal aspirate/swab for direct fluorescent antigen (DFA) detection of respiratory viruses or pertussis DNA	a) RSV b) Influenza A/B c) Parainfluenza virus d) Pertussis e) Other virus
	15. Results of viral culture from nasal aspirate/swab	a) RSV b) Influenza A/B c) Parainfluenza virus d) Other virus